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<b>(21) International Application Number:</b> PCT/US99/02852  <b>(22) International Filing Date:</b> 9 February 1999 (09.02.99)  <b>(30) Priority Data:</b> 09/040,961                      18 March 1998 (18.03.98)                      US  <b>(71) Applicant:</b> PHARMACOEPIA, INC. [US/US]; 3000 Eastpark Boulevard, Cranbury, NJ 08512 (US).  <b>(72) Inventors:</b> DAMAJ, Bassam, B.; 4121 Town Court North, Lawrenceville, NJ 08648 (US). HORLICK, Robert, A.; 29 Bradford Lane, Plainsboro, NJ 08536 (US). ROBBINS, Alan, K.; 112 South Road, Wilmington, DE 19809 (US).  <b>(74) Agents:</b> JACOBS, Seth, H. et al.; Darby & Darby P.C., 805 Third Avenue, New York, NY 10022-7513 (US).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.          Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> CO-EXPRESSION OF G <sub>ia</sub> PROTEIN AND G <sub>i</sub> PROTEIN COUPLED RECEPTOR TO ENHANCE SIGNAL TRANSDUCTION RESPONSES		
<b>(57) Abstract</b>  <p>A method for detecting compounds that bind to a G<sub>i</sub> protein coupled receptor which comprises the steps of culturing a cell in a medium under conditions wherein G<sub>ia</sub> protein and a G<sub>i</sub> protein coupled receptor are co-expressed, contacting the cell with a test compound, and assaying the cell for a cellular response to the compound, the response being an increase in the cytoplasmic calcium concentration, where the cell has been transfected with a gene encoding a G<sub>i</sub> protein coupled receptor and a gene coding for G<sub>ia</sub> protein capable of coupling to said G<sub>i</sub> protein coupled receptor. Cells useful in such methods, and methods for making such cells, are also provided.</p>		

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**CO-EXPRESSION OF  $G_{1a}$  PROTEIN AND  $G_i$  PROTEIN COUPLED RECEPTOR TO  
ENHANCE SIGNAL TRANSDUCTION RESPONSES**

**FIELD OF THE INVENTION**

10           The invention relates to methods for rapidly assaying for agonism or  
antagonism of  $G_i$  protein coupled receptors and to cells which can serve as assay  
systems for evaluating such agonism or antagonism. The invention relates particularly  
to methods for rapidly assaying for agonist and antagonist compounds which bind to  $G_i$   
15           protein coupled receptors which stimulate intracellular calcium mobilization, such as  
the nociceptin receptor, and particularly chemokine receptors such as CCR3, CCR2,  
and the interleukin 8 receptor type B.

**BACKGROUND OF THE INVENTION**

          G proteins (guanine nucleotide binding regulatory proteins) are integral  
parts of regulatory mechanisms that operate in all human cells. Impairing G protein  
20           function can affect a cell's response to hormonal signals, e.g., by interfering with  
intracellular metabolic pathways. This can cause development or maintenance of a  
wide variety of disease states.

          When functioning normally, G proteins act as essential parts of signal  
transducing mechanisms by which extracellular hormones and neurotransmitters convey  
25           their signals through the plasma membrane of the cell and thus elicit appropriate  
intracellular responses.

          These signal transducing mechanisms comprises three distinct  
components:

- 1)       a receptor protein with an extracellular binding site specific for a  
30       given agonist or hormone;
- 2)       a membrane-bound effector protein that when activated catalyzes  
the formation or facilitates the transport of an intracellular second messenger, such as  
adenylyl cyclase which converts ATP to cyclic AMP (cAMP); and
- 3)       a protein which functions as a communicator between these two.

G proteins fulfill this function as communicator between receptor and effector proteins in the generation of intracellular responses to extracellular hormones and agonists.

G proteins are composed of three polypeptide subunits: G alpha (or  $G_\alpha$ ), G beta (or  $G_\beta$ ) and G gamma (or  $G_\gamma$ ). The  $G_\beta$  and  $G_\gamma$  polypeptide subunits occur in living cells as a heterodimer, commonly referred to as a "bg" dimer. The conformation of these subunits and their degree of association with each other change during the signal transducing mechanism. These changes are associated with the hydrolysis of the nucleotide guanosine triphosphate (GTP) to form guanosine diphosphate (GDP) and free phosphate (GTPase activity). The binding sites for GTP and GDP, and the GTPase catalytic site, reside in the alpha subunit of the G protein.

An example of a G protein cycle which occurs when a signal is conveyed across the cell membrane is as follows:

In an unstimulated cell the G proteins are found in a resting state in which alpha, beta and gamma subunits are complexed together and GDP is bound to  $G_\alpha$ . The binding of an appropriate hormone or agonist to the receptor changes its conformation and causes it to activate the G protein by displacing GDP and allowing GTP to bind. This is the rate-limiting step in the G protein cycle. When GTP is bound to  $G_\alpha$  it may dissociate from bg and is able to bind to, and activate, adenylate cyclase, which releases cAMP into the cytoplasm. GTP is then hydrolysed to GDP  $G_\alpha$  and the cycle is complete.

A series of complex interactions has evolved to allow signal amplification, such that a single hormone-receptor complex can trigger the production of several hundred second messenger molecules, such as cAMP. cAMP is a potent second messenger that binds to and activates protein kinase A (PKA). PKA was first shown to play a role in glycogen metabolism and is now known to influence a variety of processes, including transcription.

This system also allows several different receptors to interact with a signal-generating enzyme. The receptors can activate the enzyme or inhibit it. For example, distinct alpha subunits  $G_{sa}$  (stimulatory) and  $G_{ia}$  (inhibitory) combine with the bg complex to form stimulatory or inhibitory G proteins. An example of a receptor that interacts with  $G_i$  to lower the concentration of cAMP is the alpha 2-adrenergic receptor. Integration of signals from  $G_s$  and  $G_i$  allows the level of cAMP in the cell to be fine-tuned in response to several different extracellular agonists.

Although G proteins were first identified and characterized in relation to the adenylyl cyclase system discussed above, it is now known that they are involved in many aspects of cell signaling. In particular, certain G proteins act in signal transducing pathways that activate phospholipase C. This enzyme catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) to form diacylglycerol (DG) and inositol 1,4,5-triphosphate (IP<sub>3</sub>). DG activates protein kinase C (PKC) which phosphorylates a certain sub-set of cellular proteins and modulates their activity. For example, PKC is important in controlling intracellular pH and in transcriptional activation of specific genes. IP<sub>3</sub> is a small water-soluble molecule that causes the release of calcium ions (Ca<sup>2+</sup>) from intracellular stores. Ca<sup>2+</sup> is a potent intracellular messenger in several metabolic and homeostatic pathways.

Many drugs are currently directed at hormone receptors. Examples include beta-adrenergic agents used in the treatment of asthma, and "beta-blockers" used in the treatment of high blood pressure. Assays for chemical compounds that act at particular G protein coupled receptors have typically been based on the use of radioligands, where either the drug of interest is radiolabeled and its binding to receptor directly assayed, or the ability of a compound of interest to displace a known radioligand from a receptor of interest is assayed. Radioligand binding assays, however, use dangerous and expensive radioisotopes, and require disposal of radioactive reagents and supplies. Also, these assays are not as amenable to high-throughput systems as are assays which monitor intracellular processes such as calcium release.

Various methods have been developed to assay for receptor/ligand interactions without using radioactive reagents. For example, to assay G protein coupled signal transduction pathways that involve the release of intracellular calcium stores, direct detection methods have been developed that precipitate calcium with alizarin sulfonate.

Receptors are normally present in very low numbers on the surfaces of cells that are useful in such assays. Therefore, when performing receptor activation assays it is common to first overexpress the receptor of interest by introducing into cells DNA which is capable of expressing the receptor.

However, merely overexpressing the desired receptor may not allow generation of sufficient signal. For example, when the receptor is activated, it may

not transduce sufficient intracellular calcium to allow unambiguous detection. This problem has been found by the present inventors to be particularly present with respect to detection of intracellular calcium release associated with the activation of  $G_i$  coupled receptors.

5           Thus, when  $G_i$  coupled receptors are overexpressed in cells, the calcium release achieved by exposure of the cells to receptor agonist or antagonist compounds can be too low for reliable measurement, i.e., the resulting signal strength may be too low for an efficient assay. Thus, there is a need for a method to increase the second messenger signal strength generated by agonist binding to receptor-overexpressing  
10 cells. The signal generated by agonist binding must be sufficiently robust that a decrease in signal, e.g., in response to antagonist binding, is detectable.

#### SUMMARY OF THE INVENTION

          It has been determined that expression of transfected DNA encoding a  $G_{ia}$  protein subunit that couples to a co-expressed receptor that couples to a  $G_i$  protein  
15 will obviate the "signal strength" problem referred to above, and will produce a significant and reproducible intracellular calcium signal that can be reliably detected.

          In one aspect, the invention relates to a method for assaying a test compound for its effect on a  $G_i$  protein coupled receptor. The method involves  
20 contacting a cell with the test compound and assaying the cell for cytoplasmic calcium concentration. According to the invention, the cell employed in such an assay has been transfected with a gene encoding a  $G_i$  protein coupled receptor and with a gene coding for a  $G_{ia}$  protein capable of coupling to the  $G_i$  protein coupled receptor to increase cytoplasmic calcium upon binding of an agonist to the  $G_i$  protein coupled  
receptor.

25           In another aspect, the present invention relates to a cell useful for high- or low-throughput assays to determine if a compound binds to a  $G_i$  protein coupled receptor, the cell comprising transfected DNA which expresses a  $G_i$  coupled receptor, transfected DNA which expresses a  $G_{ia}$  protein subunit, and an intracellular calcium release mechanism which produces a detectable signal as measurable by fluorescence  
30 of a fluorophore in response to agonist compound binding to the receptor when the  $G_i$  protein coupled receptor is coexpressed with the  $G_{ia}$  protein subunit.

          In yet another aspect, the invention relates to a method for producing a cell for detecting compounds which bind to a  $G_i$  protein coupled receptor which

comprises the steps of transfecting a cell with a gene which codes for a  $G_i$  protein coupled receptor, transfecting the cell with a gene which codes for a  $G_{ia}$  protein capable of coupling to the receptor, and culturing the cell in medium under conditions in which the  $G_{ia}$  protein and the  $G_i$  protein coupled receptor are co-expressed in the cell.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1a is a depiction of the pHEBo vector.

Figure 1b is a depiction of the p394 vector.

Figure 1c is a depiction of the pcmvmcs1 vector.

Figure 1d is a depiction of the pCDM8 vector.

Figure 1e is a depiction of the pm3ar vector.

Figure 1f is a depiction of the pm3CCR3 vector.

Figure 1g is a schematic showing the modification of the CCR3 gene by the addition of a signal sequence coding region.

Figure 1h is a depiction of the pm3CCR3sp vector.

Figure 2a is a depiction of the pE3 vector.

Figure 2b is a depiction of the pE3delta vector.

Figure 2c is a depiction of the pPur vector.

Figure 2d is a depiction of the pE3pur vector.

Figure 2e is a depiction of the pEpurG $\alpha$ 2 vector.

Figure 3 is the nucleotide sequence of the pCEP4 vector.

Figure 4 is the nucleotide sequence of the pCDM8 vector.

Figure 5 is the nucleotide sequence of the pBSIISK+ vector.

Figure 6 is the nucleotide sequence of the pPur vector.

Figure 7 is a bar graph illustrating the stability of the K $_d$  for agonist in CCR3 receptor and CCR3 receptor/G $\alpha$ 2 protein transfected cells.

Figure 8 is a bar graph illustrating the stability of the receptor number per cell in CCR3 receptor and CCR3 receptor/G $\alpha$ 2 protein transfected cells.

Figure 9 is a bar graph illustrating the increases in calcium mobilization in response to eotaxin either in cells transfected with the CCR3 receptor alone or in cells cotransfected with the CCR3 receptor and G $\alpha$ 2 protein.

Figure 10 is a bar graph illustrating the increases in calcium mobilization in response to nociceptin either in cells transfected with the nociceptin

receptor alone or in cells cotransfected with the nociceptin receptor and  $G_{i\alpha 2}$  protein.

Figure 11 is a graph representing a time course illustrating the increase in the concentration of intracellular calcium in response to the CCR2 agonist MCP-1 either in cells transfected with the CCR2 receptor alone or in cells cotransfected with the CCR2 receptor and  $G_{i\alpha 2}$  protein.

Figure 12 is graph showing the dose-response relationship for increases in cytoplasmic calcium as a result of exposure of cells to interleukin 8 either in cells transfected with the interleukin 8 receptor type B alone or in cells cotransfected with the interleukin receptor type B and  $G_{i\alpha 2}$  protein.

#### 10 DETAILED DESCRIPTION OF THE INVENTION

All patents, patent applications, and publications referred to herein are hereby incorporated by reference in their entirety. In case of a conflict in description or terminology, the present disclosure is intended to control.

##### Definitions:

15 "G<sub>i</sub> protein" as used herein refers to the heterotrimeric guanine nucleotide binding proteins G<sub>i1</sub>, G<sub>i2</sub>, and G<sub>i3</sub>, including those from any animal species (e.g. mouse), and derivatives having mutated sequences such as G<sub>ip2</sub>.

"Transfecting" as used herein means to introduce exogenous DNA into a cell.

20 "Codes for" as used herein means the property of a DNA polynucleotide to be transcribed by an RNA polymerase into messenger RNA, which messenger RNA can in turn be translated into a polypeptide. The polypeptide so produced is said to be "coded for" by the DNA polynucleotide.

25 G protein "coupling" to a receptor as used herein means the activation of a guanine nucleotide binding protein by the interaction of a cell surface receptor with an agonist for that receptor.

"Culturing" as used herein means the incubation of cells in a medium sufficient for the maintenance of cell division and cellular physiological processes.

30 "Co-expressed" as used herein means that at least two proteins are synthesized in a cell as a result of the introduction into the cell of DNA foreign to the cell.

"Cellular response" as used herein means the response of a cell to the activation of a G protein by an agonist-bound receptor, or the response of a cell to the



presence of a receptor antagonist, and includes second messenger responses such as the opening or closing of ionic channels, such as calcium or potassium channels, the activation or inhibition of adenylyl cyclase, which catalyzes the conversion of ATP to cyclic AMP, and the activation of phospholipase C, which cleaves phosphatidylinositol 4,5 bisphosphate into diacylglycerol and inositol triphosphate (IP<sub>3</sub>).

"Native DNA" of a cell or cell line is the DNA complement of a defined cell or cell line prior to the introduction, by any means, of exogenous DNA into the cell or cell line.

"Integrated" as used herein with regard to DNA refers to DNA that has been transfected into a cell which has been incorporated into the native DNA of a cell.

"Stably transfected" as used herein means the introduction into and maintenance of exogenous DNA into a cell or cell line by integration into the host chromosome or by episomal transfection for at least 2 weeks and preferably more than 4 months.

"Transiently transfected" as used herein means the introduction into and maintenance of exogenous DNA in a cell or cell line for a limited period of time. If the DNA is derived from an integrating construct, then transience is defined as 0 to 5 days post transfection or until detectable signal has faded into insignificance. If the DNA is derived from a replicating, episomal construct, then transience is defined as 0 to 21 days post transfection, or until detectable signal has faded into insignificance. A detectable signal can be the direct detection of the transfected DNA, for example by Southern blotting or PCR, or detection of an RNA transcribed from the DNA, or detection of a protein expressed by the DNA.

A "DNA construct" as used herein means a polynucleotide which contains an open reading frame which encodes a protein, and includes any promoter, transcription initiation, transcription termination, or other nucleotide sequences which might facilitate expression of the encoded protein.

An "intracellular calcium release mechanism" as used herein means any combination of cellular proteins which, when activated, function to cause the release of calcium ions from intracellular stores into the cytoplasm of the cell.

G<sub>i</sub> protein coupled receptors, such as the C-C chemokine receptor 3 (CCR3), C-C chemokine receptor 2 (CCR2), interleukin 8 receptor type B (CXCR2), and nociceptin receptor (NociR), must physically couple to G<sub>i</sub> proteins in order to

transduce extracellular stimuli into intracellular signals that lead to functional responses. We have developed an assay for detecting agonists and antagonists of  $G_i$  protein coupled receptors. The assay is particularly useful in high throughput screening to determine compounds that are useful as drug candidates.

5 When expressed from exogenously introduced DNA constructs,  $G_i$  protein coupled receptors can be expressed at low levels on the surface of the transfected cells; they may also overwhelm the endogenous cellular mechanism for transducing extracellular signals into intracellular responses. The signals generated by activation of such expressed receptors can be weak. We have determined that when  
10 performing assays relying on mobilization of calcium from intracellular stores, weakness in signal strength can be obviated by transfecting cells which express  $G_i$  protein coupled receptors with a DNA construct which encodes a  $G_{ia}$  protein subunit capable of coupling to the receptor. This can be done, for example, by cotransfecting cells with separate constructs encoding the  $G_i$  protein coupled receptor and  $G_{ia}$  protein  
15 subunit, or by transfecting a single construct which encodes both a  $G_i$  protein coupled receptor and a  $G_{ia}$  protein subunit.

Coexpression of the  $G_{ia}$  subunit has been determined to have a positive effect on intracellular calcium mobilization. It has been determined in particular that  $G_{ia}$  protein subunits transduce signal generated by  $G_i$  coupled receptors to release  
20 intracellular calcium stores. It is known that G protein  $\beta\gamma$  dimers are required in the signal transduction pathway from  $G_i$  coupled receptors to the activation of phospholipase C (which ultimately results in the release of intracellular calcium stores). See, e.g., Wu, D. et al., *Science* 261:101 (1993); Slepak, V.Z. et al., *J. Biol. Chem.* 270:4037 (1995); and Arai, H. et al., *Proc. Natl. Acad. Sci.* 94:14495 (1997). We  
25 have determined that expression of  $G_{ia}$  protein subunits can act to stimulate calcium release in response to receptor activation. Furthermore, expression of  $\beta\gamma$  dimers or phospholipase C  $\beta$  (PLC $\beta$ ) is not required.

#### DNA, VECTORS, and HOST CELLS

30 In practicing the present invention, many conventional techniques in molecular biology, microbiology, and recombinant DNA are used. Such techniques are well known and are explained fully in, for example, Sambrook et al., 1989, *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor

Laboratory Press, Cold Spring Harbor, New York; *DNA Cloning: A Practical Approach*, Volumes I and II, 1985 (D.N. Glover ed.); *Oligonucleotide Synthesis*, 1984, (M.L. Gait ed.); *Nucleic Acid Hybridization*, 1985, (Hames and Higgins); *Transcription and Translation*, 1984 (Hames and Higgins eds.); *Animal Cell Culture*, 1986 (R.I. Freshney ed.); *Immobilized Cells and Enzymes*, 1986 (IRL Press); Perbal, 1984, *A Practical Guide to Molecular Cloning*; the series, *Methods in Enzymology* (Academic Press, Inc.); *Gene Transfer Vectors for Mammalian Cells*, 1987 (J. H. Miller and M. P. Calos eds., Cold Spring Harbor Laboratory); and *Methods in Enzymology* Vol. 154 and Vol. 155 (Wu and Grossman, and Wu, eds., respectively).

10 Insertion of nucleic acids (typically DNAs) encoding the receptor and  $G_{ia}$  polypeptides into a vector to create a DNA construct for transfection is easily accomplished when the termini of both the DNAs and the vector comprise compatible restriction sites. If this cannot be done, it may be necessary to modify the termini of the DNAs and/or vector by digesting back single-stranded DNA overhangs generated  
15 by restriction endonuclease cleavage to produce blunt ends, or to achieve the same result by filling in the single-stranded termini with an appropriate DNA polymerase.

Alternatively, any restriction site desired may be produced, e.g., by ligating nucleotide sequences (linkers) onto the termini. Such linkers may comprise specific oligonucleotide sequences that define desired restriction sites. Restriction sites  
20 can also be generated by the use of the polymerase chain reaction (PCR). See, e.g., Saiki *et al.*, 1988, *Science* 239:48. The cleaved vector and the DNA fragments may also be modified if required by homopolymeric tailing.

Nucleic acids utilized in the invention may be isolated directly from well-known cells. Alternatively, the polymerase chain reaction (PCR) method can be  
25 used to produce the nucleic acids of the invention, using either chemically synthesized strands or genomic material as templates. Primers used for PCR can be synthesized using the sequence information provided herein and can further be designed to introduce appropriate new restriction sites, if desirable, to facilitate incorporation into a given vector for recombinant expression. A clone containing DNA expressing  $G_{ia2}$  is  
30 available from the American Type Culture Collection, Rockville, MD, Cat. No. 63311, and the sequence of  $G_{ia2}$  protein is available from Genbank, accession number M13963. The sequence of a  $G_{ia1}$  protein is available from Genbank, accession number M17219, and the sequence of a  $G_{ia3}$  protein is also available from Genbank, accession

number M20597. A clone containing DNA expressing  $G_{ip2}$  is available from the American Type Culture Collection, Rockville, MD, Cat. No. 63312; the DNA has the sequence  $G_{ia2}$ Q205L.

5 The nucleic acids employed in the present invention may be flanked by native regulatory sequences, i.e., those that are found associated with the relevant gene in the genome of the organism from which the gene is isolated, or may be associated with heterologous sequences, including promoters, enhancers, response elements, signal sequences, polyadenylation sequences, introns, 5'- and 3'- noncoding regions, and the like. The nucleic acids may also be modified by many means known in the art. Non-limiting examples of such modifications include methylation, "caps",  
10 substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoroamidates, carbamates, etc.) and with charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.).

15 The invention employs nucleic acid vectors, or DNA constructs comprising nucleic acid sequences which code for the  $G_{ia}$  protein or  $G_i$  coupled receptors. It is understood that if desired, functional derivatives or fragments thereof may be employed to the same effect.

A large number of well-known vectors can be used in the invention for  
20 expression in a variety of eukaryotic hosts. For example, the  $G_{ia}$  protein or  $G_i$  coupled receptors may be expressed by transfecting known mammalian expression vectors, such as vectors available commercially from, e.g., Invitrogen Corporation, Carlsbad, CA, such as pcDNA1.1 for transient transfection, and pcDNA3.1, pcDNA3.1/zeo or pcDNA3.1/hyg, pRc/RSV or pRc/CMV2 for stable transfection, employing appropriate  
25 eukaryotic host cells, and using methods disclosed herein or otherwise known to those skilled in this art.

Recombinant cloning vectors employed in the invention can include one or more replication systems for cloning or expression, one or more markers for selection in the host, e.g. antibiotic resistance, and one or more expression cassettes.  
30 The inserted  $G_{ia}$  protein and  $G_i$  coupled receptor coding sequences may be synthesized by standard methods, isolated from natural sources, or prepared as hybrids. Ligation of the  $G_{ia}$  protein and  $G_i$  coupled receptor coding sequences to transcriptional regulatory elements and/or to other amino acid coding sequences is achieved by known

methods. Suitable host cells may be transformed/transfected/infected as appropriate by any suitable method including electroporation,  $\text{CaCl}_2$  mediated DNA uptake, microinjection, and microprojectile based transfection.

5 A large number of transcription initiation and termination regulatory regions that can be employed in the invention have been isolated by those skilled in this art and shown to be effective in the transcription and translation of heterologous proteins in eukaryotic host cells. These regions, methods of isolating them and ways to manipulate them are well-known in the art.

10 Advantageously, vectors and DNA constructs employed in the invention also include a transcription regulatory element (i.e., a promoter) operably linked to the nucleotide sequence encoding the protein to be expressed. The promoter may optionally contain operator portions and/or ribosome binding sites. Suitable promoters for mammalian cells include without limitation viral promoters such as those from Epstein-Barr Virus (EBV), Simian Virus 40 (SV40), Rous sarcoma virus (RSV),  
15 adenovirus (ADV), and bovine papilloma virus (BPV). Mammalian cells may also require terminator sequences and poly(A) addition sequences. Enhancer sequences, which increase expression, may also be included. Sequences which cause amplification of the gene may also be desirable. These sequences are well described in the art.

20 Nucleic acids encoding  $G_{ia}$  or  $G_i$  coupled receptor polypeptides may also be introduced into cells by known recombination methods. For example, such nucleic acids can be introduced into a cell to effect homologous recombination at the site of a corresponding endogenous gene or a sequence with substantial identity to the gene. Other known recombination-based methods such as nonhomologous recombination or deletion of endogenous genes by homologous recombination may also be used.

25 Preferably, separate episomal plasmids are used for expression of the  $G_i$  coupled receptor and the  $G_{ia}$  protein subunit. Each such episome preferably contains its own selectable marker. The two episomes may be co-transfected simultaneously into the same cell to produce a pool of stably transformed cells available for use in high-throughput screening assays. Such cells are advantageously obtained within about  
30 2 weeks.

Transformation methods in which either the gene encoding the  $G_i$  coupled receptor or the gene encoding the  $G_{ia}$  subunit (or both) is integrated into the host nuclear material are less preferred, as the time required to obtain a cell line in this

manner which stably expresses either protein is normally from three to six months. Using the preferred episomal expression system of the invention, the need for clonal selection of stably transfected cells is eliminated, assayable cell lines are generated in weeks rather than months, and the cellular response achievable in response to receptor  
5 activation is significantly enhanced.

The preferred episomal expression system of the invention employs episomes bearing an EBV origin of replication in a permissive cell type expressing the EBNA1 protein for recombinant receptor binding assays. Primate and canine cell lines are preferred as host cells. Rodent cells are not preferred since they are not  
10 permissive for EBNA1/EBV oriP interactions, i.e., rodent cells do not permit replication of EBV oriP based vectors.

HEK-293 cells are particularly preferred and are available from the American Type Culture Collection (ATCC), Rockville, MD, Accession Number CRL-1573. HEK-293 cells that constitutively express EBNA1 are most preferred, and  
15 are commercially available from Invitrogen Corporation, Carlsbad, CA.

If desired, episomes can be used for the construction of integratively stable cell lines that express EBNA1. That is, the episomes of the invention can be used to transfect cells, and rather than replicate independently, the genes encoded on the episomes can be integrated into the chromosomes of the host cell, thereby allowing  
20 replication along with the host cell's DNA. If episomes are employed which also express the EBNA1 protein, then any permissive cell line can be used to practice the invention, regardless of whether or not it pre-expresses EBNA1.

The preferred episomally co-transfected cell lines of the invention remain stably transfected for at least 5 months after transfection, preferably as  
25 demonstrated by ligand-induced cellular responses, northern blot analysis of steady state RNA levels (encoding receptor and G protein), and western blot analysis of G protein levels.

In order to detect the release of intracellular calcium stores in response to stimulation of  $G_i$  coupled receptors by agonists, or the inhibition of such release due  
30 to binding of antagonists to  $G_i$  coupled receptors, both direct and indirect measurements can be used. Such methods are well-known in this art. For example, direct detection methods are well-known that precipitate calcium with alizarin sulfonate. Other well-known direct detection methods use photoproteins that fluoresce

when bound to calcium, such as aequorin and obelion. Recently, non-protein fluorescent indicators of calcium release have been developed for use in direct detection; these include Fura-2/AM, Indo-1/AM, and Fluo-3/AM and their derivatives. Such fluorescent probes are commercially available, and can be obtained from

5 Molecular Probes, Eugene, OR; Teflabs, Texas; and Sigma Chemical Co., St. Louis, MO.

Other known methods for direct detection of calcium use metallochromic indicators such as murexide, arsenazo III, and antipyrilazo III.

Calcium release can be indirectly measured using patch clamps (which

10 record calcium release in single cells), mini/microelectrodes, and vibrating calcium electrodes.

Confocal microscopy permits sensitive visual detection of fluorescence due to calcium. Methods of screening using confocal microscopy are described in U.S. patent application Serial No. 08/868,280 filed June 3, 1997.

15 The method of the invention can be carried out as part of a high throughput screening of a library of compounds for binding to receptor. In one embodiment of the invention, the method is carried out with a plurality of compounds to be screened, preferably at least about 96 compounds, such as when using a 96 well microtitre plate. Such assays can also be performed in the 1536 well plate described in

20 U.S. patent application serial no. 60/037,636, filed February 18, 1997. The library of compounds to be screened can be quite large, e.g., containing more than 100,000 compounds.

It is preferred that the compounds assayed in the high throughput method be derived from combinatorial libraries on polymer beads. By synthesizing

25 sufficient compound on each bead for a few assays, compound handling is reduced or eliminated.

Preferably, the library compounds are eluted from the beads and evaporated to dryness in microtiter plates in preparation for the assay. Compounds on beads can be released by photocleavage, or another type of cleavage. Cleavage of

30 photocleavable linkers is preferred. Such linkers, and methods for their cleavage, are described in Barany et al. (1985) *J. Am. Chem. Soc.* 107:4936. Examples of other linkers and the relevant cleavage reagents are described in WO 94/08051.

Using combinatorial libraries prepared on beads, the identity of active

compounds is preferably determined using the encoding system described in WO 94/08051 and in WO 95/30642. In this system, chemical tags encoding the identities of the compounds are applied to the solid supports. The identity of the compound on a given support can be determined by detaching the chemical tags from the support, identifying the tags by, e.g., gas chromatography, and correlating the identities of tags with the identity of the compound. Once an active compound is identified, the corresponding bead (which had contained the compound) can be examined, and the identity of the compound determined by releasing the tags and decoding by this method.

The following examples are intended to illustrate the invention only, and are not intended to be limiting in any way. Many variations and adaptations of the present invention will be apparent to those of ordinary skill in the art, and these are intended to be included within the scope of the invention.

#### **EXAMPLE 1: CONSTRUCTION OF EPISOMAL EXPRESSION VECTORS**

##### **A. Construction of pHEBO Vector**

The pHEBo vector was made using commercially available vectors. The sequence of vector pBR322 (Genbank accession number synpbr322) from nucleotide 1 to nucleotide 772 was ligated to the nucleotide sequence of vector pCEP4, SEQ ID NO: 1, Figure 3, from position 8146 to 10376 (Invitrogen, Carlsbad, CA, Cat. No. V004-50). To this construct was ligated pCEP4 nucleotides 1333 to 5500. Prior to ligation, fragments were PCR amplified or joined using preexisting restriction sites. The resulting plasmid contained the Epstein Barr Virus (EBV) origin of replication (oriP), a hygromycin resistance marker (hyg) transcribed from the minimal Herpes Simplex Virus (HSV) thymidine kinase (tk) promoter, and was terminated with the tk poly adenylation signal (poly(A)), in vector pBR322. The pHEBo vector is shown schematically in Figure 1a.

##### **B. Construction of pcmvmcs1 Vector**

Vector p394 was constructed according to Colberg-Poley, A.M. et al. *J Virol.* 1992 Jan; 66(1): 95-105. Briefly, the vector can be made by cloning the CMV IE promoter (which can be obtained from vector pCEP4, SEQ ID NO: 1, nucleotide 1132 to 474) into the EcoRV site of pBSIISK(+) SEQ ID NO: 6. Oligonucleotides 5'-ATATCATAATATGTACATTTATATTG-3', SEQ ID NO: 13 and 5'-TCGCGACGTCTCCGTGTAGGCGATCTGACGGTTCCTAAAC-3', SEQ ID NO:



14 were used to amplify the promoter.

The SV40 poly(A) signal, which can be obtained, e.g., from pCEP4, SEQ ID NO: 1 (from the native BsaBI site at nucleotide 176 to the native BamHI site at position 412) was cloned into the SmaI and BamHI sites of pBSSK(+)-CMVIE.

- 5 Using the remaining EcoRI and PstI sites in between the CMV promoter and SV40 poly(A), a multicloning site was added using oligonucleotides:

5'-AATTCGCGACGCGTGATATCTGCAGGCCTAGATCTCTAGATAAGTAAT  
GATCATGCA-3', SEQ ID NO: 15

and

- 10 5'-TGATCATTACTTATCTAGAGATCTAGGCCTGCAGATATCACGCGTCGCG-3',  
SEQ ID NO: 2, yielding vector p394.

Vector p394 (Figure 1b), was cleaved with HindIII and BamHI to yield a 1.3 kb HindIII - BamHI fragment containing the cytomegalovirus immediate early promoter (CMV), a multicloning site region (mcs), and the SV40 poly(A) region.

- 15 This fragment, which comprises an "expression cassette" was cloned into the HindIII and BamHI sites of pHEBo to yield pcmvmcs1 (Figure 1c). The mcs contains the following restriction enzyme sites: Esp3I, EcoRI, NruI, MluI, EcoRV, PstI, StuI, BglII. The mcs in vector pcmvmcs1 was replaced with the following sites: Esp3I, AgeI, StuI, KpnI, AvrII, XhoI, by a synthetic oligonucleotide linker that contained  
20 overhangs compatible with the Esp3I and BglII sites. The BglII site was not recreated by the oligonucleotide linker (Figure 2e). This vector was designated pcmvmcs3.

### C. Construction of pm3ar Vector

- An intron (called IVS or "intervening sequence") was added to the expression cassette (defined herein as the CMVIE-mcs-poly(A) containing nucleotides)  
25 as follows. A XhoI - BamHI fragment containing the SV40 early intron and poly(A) signals was excised from vector pCDM8 (Invitrogen, Carlsbad, CA; Figures 1d and 4), SEQ ID NO: 3. The poly(A)-containing fragment was removed from vector pcmvmcs3 by digestion with restriction enzymes XhoI and BamHI, and the XhoI-BamHI fragment from pCDM8 was added, generating vector pm3ar (Figure 1e).

### 30 D. CCR3 Expression Vector

An episomal vector which codes for the C-C chemokine receptor 3 ("CCR3") was constructed. The coding region for the receptor was obtained by PCR amplification of genomic DNA, using the oligonucleotide 5'-

GTGAAATGACAACCTCACTAGATACAG-3', SEQ ID NO: 4 as the sense primer, and 5'-CTGACCTAAAACACAATAGAGAGT-3', SEQ ID NO: 5, as the antisense primer. The PCR fragment obtained was cloned into the EcoRV site of pBSIISK+ (Figure 5)(a Bluescript vector commercially available from Stratagene, La Jolla, CA, 5 Stratagene Cat. No. 212205, Genbank accession number 52325), SEQ ID NO. 6.

The coding region was excised from pBSIISK+ using the restriction enzymes SpeI and NsiI, and the fragment containing DNA coding for CCR3 was cloned into the AvrII and Sse8387I sites of vector pm3ar (Figure 1e) to generate episomal expression construct pm3CCR3 (Figure 1f).

10 A hydrophobic signal sequence was added to the CCR3 coding sequence by PCR (see Figure 1g). Vector pm3CCR3 was used as a template and oligonucleotide 144, 5'-TGTCGATTGTCAGCAGGATTATG-3' SEQ ID NO: 7 (which begins at nucleotide +390 and maps 3' to the unique BglII restriction site on the vector) and oligonucleotide 143, 5'-GTTCTGTCTCTGCTGCCACTG

15 CTCGAGGCTCAAACAACCTCACTAGATACAGTTGAG-3', SEQ ID NO: 8 (which overlaps the CCR3 coding sequence and contains a long tail encoding approximately two-thirds of the hydrophobic signal sequence) were used as primers. The resulting 428 base pair fragment was then used as a template for PCR, using oligonucleotide 144, SEQ ID NO: 7 and oligonucleotide 142, GAGCAGCCGGCACC

20 ACCATGGCTCTGTCTTGGGTTCTGACTGTTCTGTCTCTGCTGCCACTG, SEQ ID NO: 9 (which encodes the remainder of the hydrophobic signal sequence and contains a Kozak consensus sequence for efficient initiation of translation). The resulting 461 base pair fragment was digested with NgoMI and BglII and cloned into the AvrII and BglII sites of pm3CCR3 to generate expression vector pm3CCR3sp

25 (Figure 1h).

#### E. Construction of pE3 Vector

Vector pm3ar (Figure 1e) was altered to provide an additional set of cloning sites immediately upstream from the CMVIE promoter. The new sites were added using a synthetic oligonucleotide linker 5'-

30 CGATCACGTGCAGCTGAGATCTA-3', SEQ ID NO: 10, that contained the restriction sites, ClaI, AscI, BssHII, PacI, HindIII and overhangs compatible with the ClaI and HindIII sites of pm3ar. The new vector was designated pE3 (Figure 2a).

#### F. Construction of pE3delta Vector

Vector pE3delta (Figure 2b) was generated by the digestion of vector pE3 with BstBI and BspLU11I to remove the hygromycin coding region. The hygromycin coding region was replaced with a synthetic oligonucleotide linker 5'-CATGTAGATCTCAGCTGCACGTGAT-3', SEQ ID NO: 11 containing multiple cloning sites.

#### G. Construction of pE3pur Vector

Vector pE3pur (Figure 2d) was constructed by the digestion of vector pE3delta with PvuII and BspLU11I followed by ligation to a PvuII - BglIII fragment (Figure 2c) obtained from vector pPur (Clontech, Cat. No. 6156-1, Genbank accession number U07648, SEQ ID NO: 12, Figure 6). The PvuII - BglIII fragment from vector pPur contains the SV40 promoter, a puromycin resistance gene, and an SV40 poly(A) tail.

#### H. $G_{i\alpha 2}$ Expression Vector

Vector pBN31, which contains the wildtype sequence for murine  $G_{i\alpha 2}$  cloned into the EcoRI site of vector pCDNAI, was obtained from the American Type Culture Collection (ATCC), Cat. No. 63311, Rockville, MD. pE3pur vector (Figure 2d) was digested with KpnI and XhoI, which correspond to restriction sites found within the multicloning regions at the 5' and 3' ends, respectively, of the  $G_{i\alpha 2}$  coding region. The vector obtained from the ATCC was also digested with KpnI and XhoI, and a fragment containing the  $G_{i\alpha 2}$  coding region was excised. This fragment was cloned into the KpnI and XhoI sites of vector pE3pur, to produce vector pEpurG $i\alpha 2$  (Figure 2e). This vector was used without further modification to transfect cells.

#### EXAMPLE 2: TRANSFECTION OF CELLS AND MAINTENANCE OF STABLY TRANSFECTED CELL LINES

293E cells (HEK-293 cells which constitutively express the Epstein-Barr virus nuclear antigen-1, commercially available from Invitrogen Corp., Carlsbad, CA, cat. no. R620-07) were transfected using the calcium phosphate or lipofectamine procedures as described in Sambrook et al., (1989) "Molecular Cloning: A Laboratory Manual," Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 1633-1634.

T75 flasks containing approximately  $1 \times 10^6$  293E cells were transfected with plasmid p3ARccr3 with or without cotransfection with pEpurG $i\alpha 2$ . 5 mg of each plasmid was used in the transfection reaction, with 62 ml of 2 M  $\text{CaCl}_2$  in enough

water to achieve a final volume of 500 ml. To this solution was added 500 ml of HEPES-buffered saline ("HBS") and the entire 1 ml of solution was added directly to the T75 culture medium (12 ml of Dulbecco's Modified Eagle Medium, "DMEM", supplemented with 10% fetal bovine serum, penicillin 100 units/ml, streptomycin 100 mg/ml, glutamine 2 mM, hereinafter called "complete DMEM"). The transfection mix was allowed to remain on the cells for 48 hours at which time the cells were washed once with phosphate buffered saline and re-fed selective media (complete DMEM supplemented with 1 mg/ml puromycin and 250 mg/ml hygromycin, hereinafter called "selective media"). The selective media was changed at day 5 post-transfection (i.e., day 5 after the transfection mix was removed) and again when the cells approached confluence, at which time cells were routinely split, i.e., "passed" between 1:4 and 1:50 in fresh selective media as needed. Frozen stocks of cells were made at cell passage 3.

### EXAMPLE 3: STABILITY OF EXPRESSION FROM EPISOMES

Cells expressing either the CCR3 receptor, or both the CCR3 receptor and  $G_{i2}$  protein, generated according to Example 2, were assayed for agonist binding characteristics. The cells were incubated in  $^{125}$ I-eotaxin at concentrations ranging from 45 pM to 300 nM. Specific binding was determined at each concentration, as was the maximum binding for a given number of cells. Based on these values, the  $K_d$  for eotaxin was determined at the end of 1 to 5 months after vector transfection. As can be seen from Figure 7, the  $K_d$  for the cells was stable, remaining between 0.2 and 0.3 nM for the entire five months for the cotransfected cells, and between 0.2 and 0.5 nM for the cells containing CCR3 receptors which were not coexpressed with  $G_{i2}$ .

In addition to the stability observed for  $K_d$  values, the total receptor number was also stable over the five month period, regardless of whether the CCR3 receptor was coexpressed with  $G_{i2}$  protein or not. In particular, Figure 8 shows that the receptor number in the singly transfected and cotransfected cells was approximately equal and stable over the entire 5 month assay period, at about 20,000 receptors per cell.

The stability of the  $K_d$  of the receptors for agonist over time is important, as variations in  $K_d$ , particularly increases in  $K_d$ , could lead to false negative responses in assays for compounds that bind to receptors.

#### EXAMPLE 4: ASSAYS FOR CALCIUM MOBILIZATION IN RESPONSE TO RECEPTOR BINDING OF AGONIST COMPOUNDS

Assays were conducted utilizing the methods described in S.R. McColl and R.H. Naccache, *Methods in Enzymology* 288:301-309, 1997, "Calcium Mobilization Assays". Cell suspensions ( $10^7$  cells/ml) were loaded with the fluorescent probe Fluo-3/AM (Teflabs, Texas) at 2 mM for 60 minutes at room temperature. The cells were washed, resuspended at  $2-4 \times 10^6$  cells/ml in Hanks' Buffered Salt Solution, made 1.6 mM in  $\text{CaCl}_2$  and 10 mM in HEPES (HBSS). Mock transfected cells, cells transfected with the CCR3 receptor only, cells transfected with  $G_{i\alpha 2}$  protein only, and cells cotransfected with CCR3 receptor and with  $G_{i\alpha 2}$  protein were then incubated in medium which comprised 100 nM of the CCR3 receptor agonist eotaxin. The cells' fluorescence was measured in a spectrofluorometer (Model SLM, Bowman Series 2, SLM-Aminco, Champagne, IL) at both the excitation wavelength, i.e., 506 nm, and the emission wavelength, i.e., 526 nm, for the chromophore. Internal calcium concentrations were calculated as described in Tsien, et al., *J. Cell. Biol.* 94:3325 (1982). Each assay was individually calibrated.

Upon addition of receptor agonist, a nearly four-fold increase in fluorescence was detected in the cells which co-expressed the CCR3 receptor and  $G_{i\alpha 2}$  protein compared to the fluorescence achieved in the cells transfected with the CCR3 receptor alone (Figure 9). No calcium increases were observed in mock-transfected cells or in cells transfected with the G-proteins alone upon stimulation with eotaxin. The data demonstrate that the episomally expressed receptor/G-protein alpha subunit signal transduction apparatus is useful as early as two weeks post transfection, and is stable for at least 20 weeks.

A similar experiment was performed using the nociceptin receptor. The results shown in Figure 10 demonstrate that upon addition of nociceptin receptor agonist, a two-fold increase in fluorescence was detected in the cells which co-expressed the nociceptin receptor and  $G_{i\alpha 2}$  protein compared to the fluorescence achieved in the cells transfected with the nociceptin receptor alone. Again, receptor and G protein alpha subunit expression from episomes provided for readily assayable calcium responses in as little as two weeks.

Figure 11 shows the increase in calcium flux that can be obtained in cells cotransfected with the CCR2 receptor and  $G_{i\alpha 2}$  relative to the calcium release

obtainable in cells transfected with only the CCR2 receptor in response to the chemokine MCP-1 (30 nM). The experiment was performed 2 months after transfection or cotransfection of cells. As can be seen from Figure 11, calcium release in cotransfected cells in response to exposure to CCR2 agonist MCP-1 was

5 significantly greater than in cells which only expressed the CCR2 receptor.

Calcium release in response to increasing concentrations of interleukin 8 was evaluated in cells expressing either the interleukin 8 receptor type B alone, or the interleukin 8 receptor type B coexpressed with  $G_{i2}$ . The cells had been transfected 1 month prior to the assay. As can be seen from the data presented in Figure 12, there

10 was an increase in the amount of calcium released from the cotransfected cells relative to the calcium released by the cells which were transfected only with the interleukin 8 receptor type B. These data are consistent with those presented for the CCR3, CCR2, and nociceptin receptors.

Thus, these data demonstrate that coexpression of  $G_i$  alpha proteins with

15  $G_i$  protein coupled receptors provides for consistent increases in calcium signaling, allowing for more sensitive and reliable assays for receptor binding compounds, i.e., agonists and antagonists.

**WHAT IS CLAIMED IS:**

1. A method for assaying a test compound for its effect on a  $G_i$  protein coupled receptor which comprises the steps of:  
contacting said cell with a test compound; and  
assaying said cell for calcium concentration in the cytoplasm of said cell;  
said cell having been transfected with a gene encoding a  $G_i$  protein coupled receptor and with a gene coding for  $G_{ia}$  protein capable of coupling to said  $G_i$  protein coupled receptor to increase said cytoplasmic calcium upon binding of an agonist to said  $G_i$  protein coupled receptor.
2. The method of claim 1 wherein said test compound is a receptor antagonist.
3. The method of claim 1 wherein said test compound is a receptor agonist.
4. The method of claim 1 wherein said gene coding for a  $G_{ia}$  protein codes for  $G_{ia2}$ .
5. The method of claim 1 wherein said gene coding for a  $G_{ia}$  protein codes for  $G_{ia1}$  or  $G_{ia3}$ .
6. The method of claim 1 wherein said gene coding for a  $G_i$  protein coupled receptor and said gene coding for a  $G_{ia}$  protein capable of coupling to said receptor are expressed from separate DNA constructs.
7. The method of claim 1 wherein said gene coding for a  $G_i$  protein coupled receptor and said gene coding for a  $G_{ia}$  protein capable of coupling to said receptor are expressed from a single DNA construct.
8. The method of claim 1 comprising direct measurement of said cytoplasmic calcium concentration within said cell.

9. The method of claim 1 comprising indirect measurement of said cytoplasmic calcium concentration within said cell.
10. The method of claim 1 wherein said gene coding for said  $G_i$  protein coupled receptor is integrated in chromosomal DNA of said cell.
11. The method of claim 1 wherein said gene coding for said  $G_i$  protein coupled receptor is expressed by a stably transfected episomal expression vector.
12. The method of claim 1 wherein said gene coding for said  $G_i$  protein coupled receptor is expressed by a transiently transfected expression vector.
13. The method of claim 1 wherein said gene coding for said  $G_{ia}$  protein is integrated in said native DNA of said cell.
14. The method of claim 1 wherein said gene coding for said  $G_{ia}$  protein is expressed by a stably transfected episomal expression vector.
15. The method of claim 1 wherein said gene coding for said  $G_{ia}$  protein is expressed by a transiently transfected expression vector.
16. The method of claim 11 wherein said gene coding for said  $G_{ia}$  protein is expressed by a stably transfected episomal expression vector.
17. The method of claim 16 wherein said  $G_i$  protein coupled receptor is selected from the group consisting of the C-C chemokine receptor 3, the C-C chemokine receptor 2, the interleukin 8 type B receptor, and the nociceptin receptor.
18. The method of claim 16 wherein said assaying comprises:  
detecting fluorescence produced by said cell;  
said cell having been loaded with a fluorophore that fluoresces in the presence of calcium.



19. The method of claim 18 wherein said fluorophore is Fura-2/AM, Indo-1/AM, or Fluo-3/AM.

20. The method of claim 1 wherein said cell has not also been transfected with genes encoding  $G_{i\beta}/G_{i\gamma}$  dimer or phospholipase C  $\beta$ .

21. The method of claim 1 wherein said test compound is a receptor antagonist and is contacted with said cell in the presence of a receptor agonist, and wherein said step of contacting decreases said calcium concentration as compared with the calcium concentration of said cells contacted with said receptor agonist alone.

22. A cell useful for assaying a test compound for its effect on a  $G_i$  protein coupled receptor, said cell comprising:  
transfected DNA which expresses a  $G_i$  coupled receptor;  
transfected DNA which expresses a  $G_{i\alpha}$  protein subunit; and  
an intracellular calcium release mechanism which produces a detectable signal as measured by fluorescence of a fluorophore in response to agonist compound binding to said receptor when said  $G_i$  protein coupled receptor is coexpressed with said  $G_{i\alpha}$  protein subunit.

23. The cell of claim 22 wherein said DNA which expresses a  $G_i$  coupled receptor is exogenous DNA that has been stably integrated into chromosomal DNA of said cell.

24. The cell of claim 22 wherein said DNA which expresses a  $G_i$  coupled receptor is contained in a stably transfected episomal expression vector.

25. The cell of claim 22 wherein said DNA which expresses a  $G_i$  coupled receptor is contained in a transiently transfected episomal expression vector.

26. The cell of claim 22 wherein said DNA which expresses a  $G_{i\alpha}$  protein subunit is exogenous DNA stably integrated into chromosomal DNA of said cell.

27. The cell of claim 22 wherein said DNA which expresses a  $G_{ia}$  protein subunit is contained in a stably transfected episomal expression vector.

28. The cell of claim 22 wherein said DNA which expresses a  $G_{ia}$  protein subunit is contained in a transiently transfected expression vector.

29. The cell of claim 22 wherein said  $G_i$  protein coupled receptor is selected from the group consisting of the C-C chemokine receptor 3, the C-C chemokine receptor 2, the interleukin 8 type B receptor, and the nociceptin receptor.

30. The cell of claim 22 wherein said fluorophore is Fura-2/AM, Indo-1/AM, or Fluo-3/AM.

31. The cell of claim 22 which has not also been transfected with genes encoding  $G_{i\beta}$ / $G_i$  dimer or phospholipase C  $\beta$ .

32. A method for producing a cell for detecting compounds which bind to a  $G_i$  protein coupled receptor which comprises the steps of:

transfecting a cell with a gene which codes for a  $G_i$  protein coupled receptor;

transfecting said cell with a gene which codes for a  $G_{ia}$  protein capable of coupling to said receptor; and

culturing said cell in medium under conditions in which the  $G_{ia}$  protein and the  $G_i$  protein coupled receptor are co-expressed in said cell.

33. The method of claim 32 wherein said gene coding for a  $G_i$  protein coupled receptor and said gene coding for a  $G_{ia}$  protein capable of coupling to said receptor are each expressed from a different DNA construct.

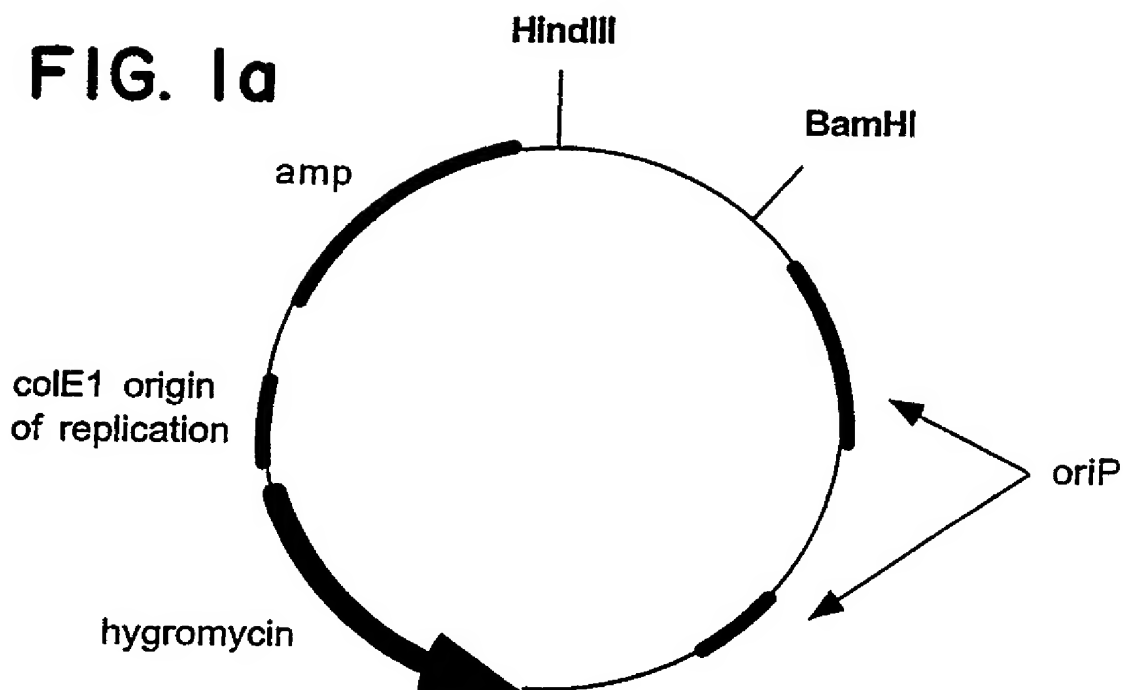
34. The method of claim 32 wherein said gene coding for a  $G_{ia}$  protein codes for a member selected from the group consisting of  $G_{ia1}$  and  $G_{ia3}$ .

35. The method of claim 32 wherein said gene coding for a  $G_{ia}$

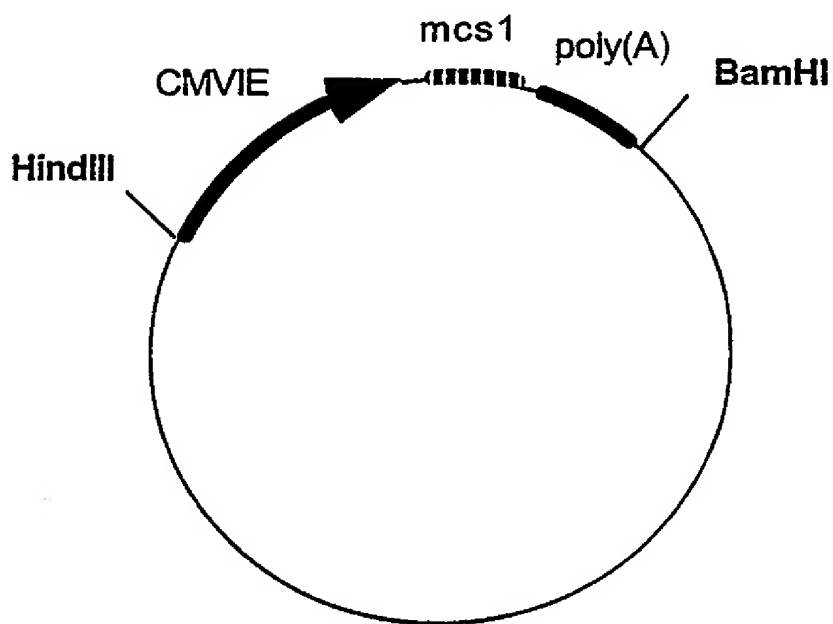
protein codes for  $G_{i2}$ .

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**FIG. 1a**



**FIG. 1b**



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FIG. 1c

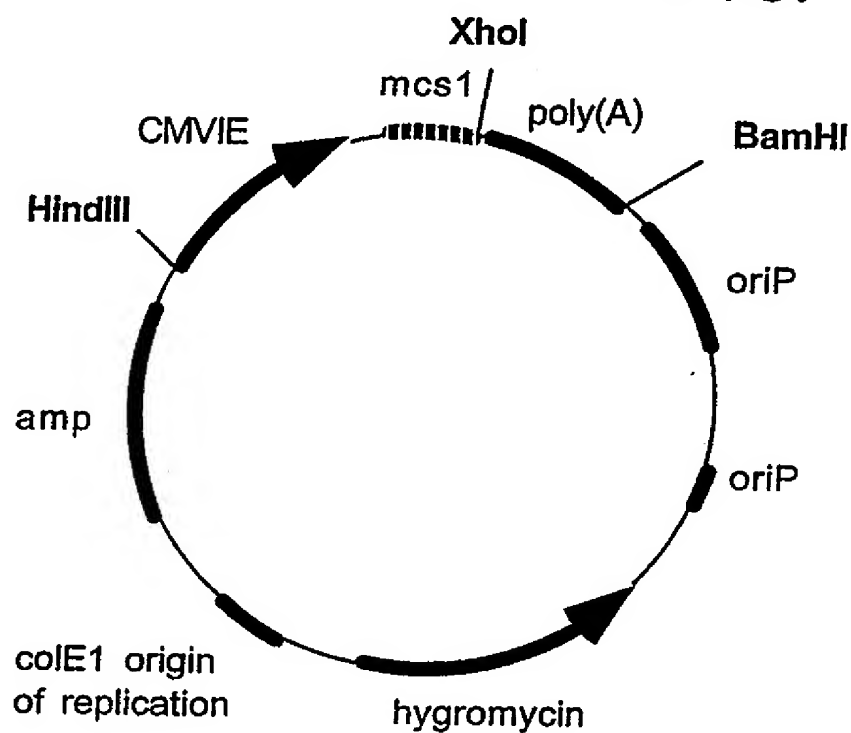
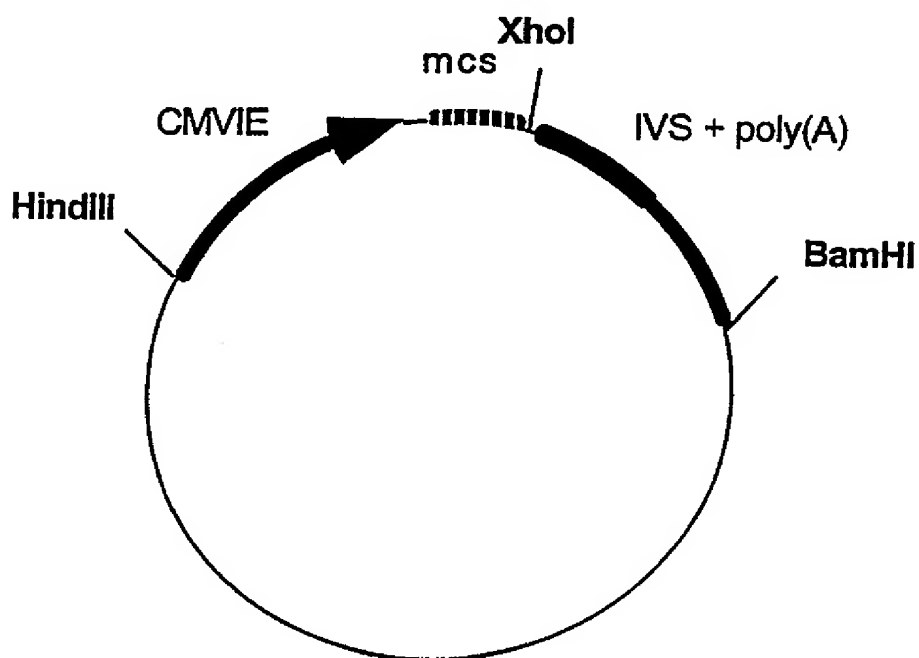


FIG. 1d



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FIG. 1e

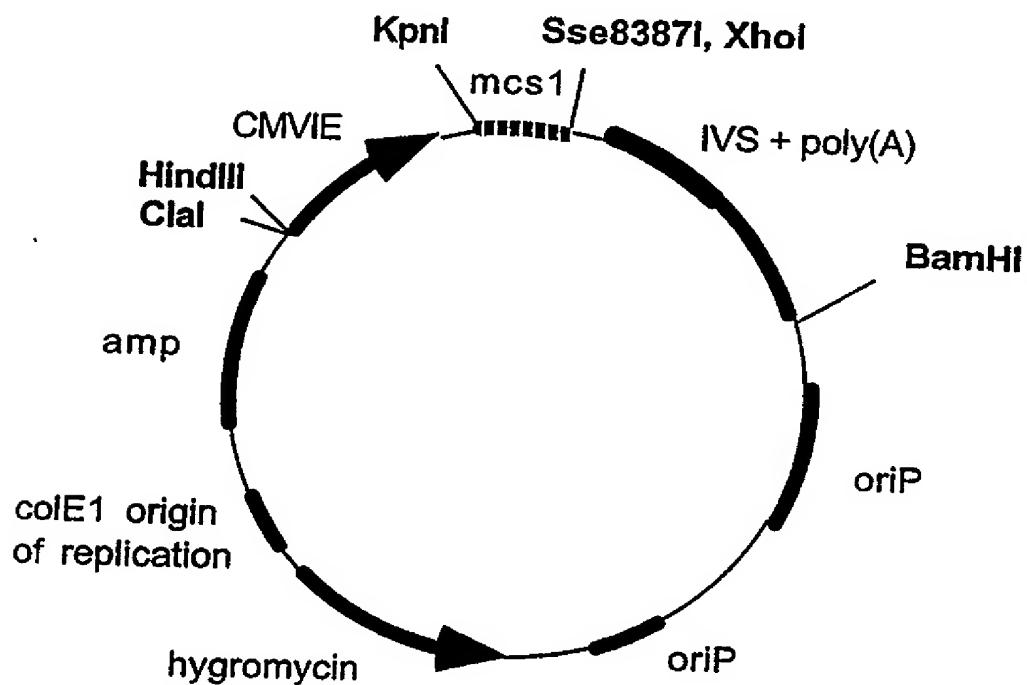
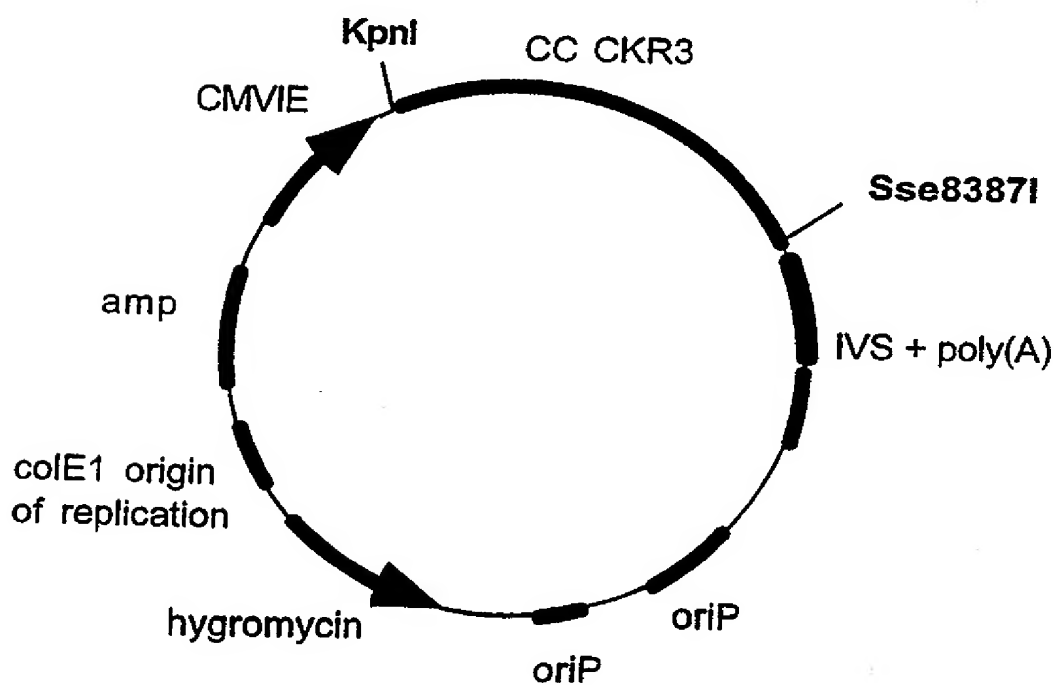


FIG. 1f



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FIG. 1g

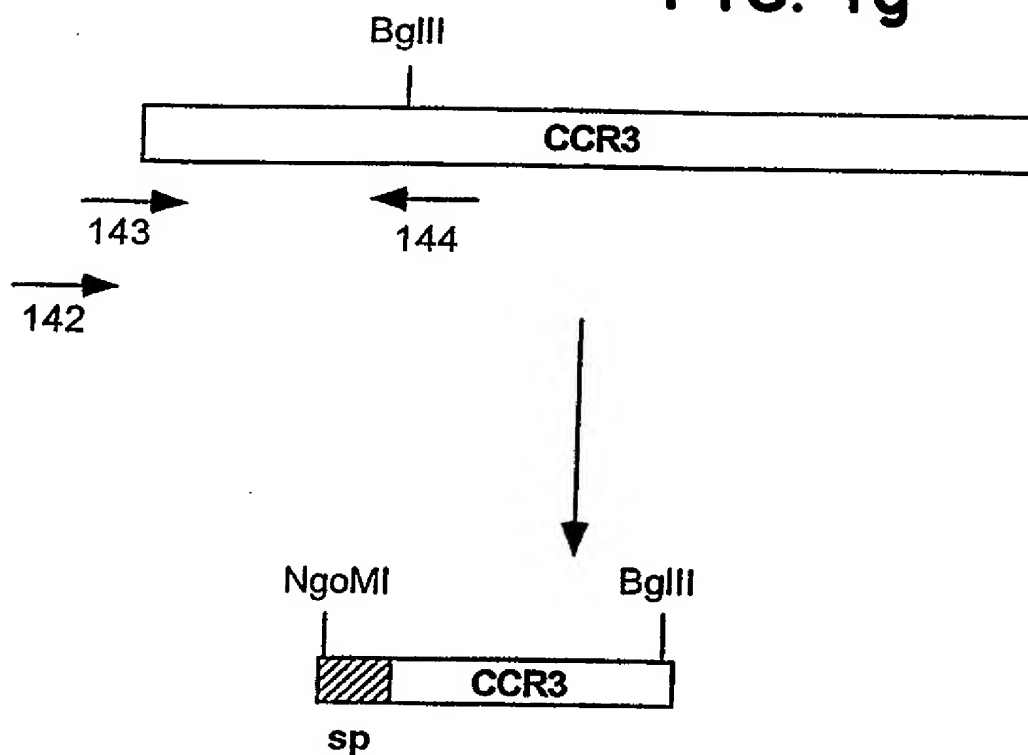
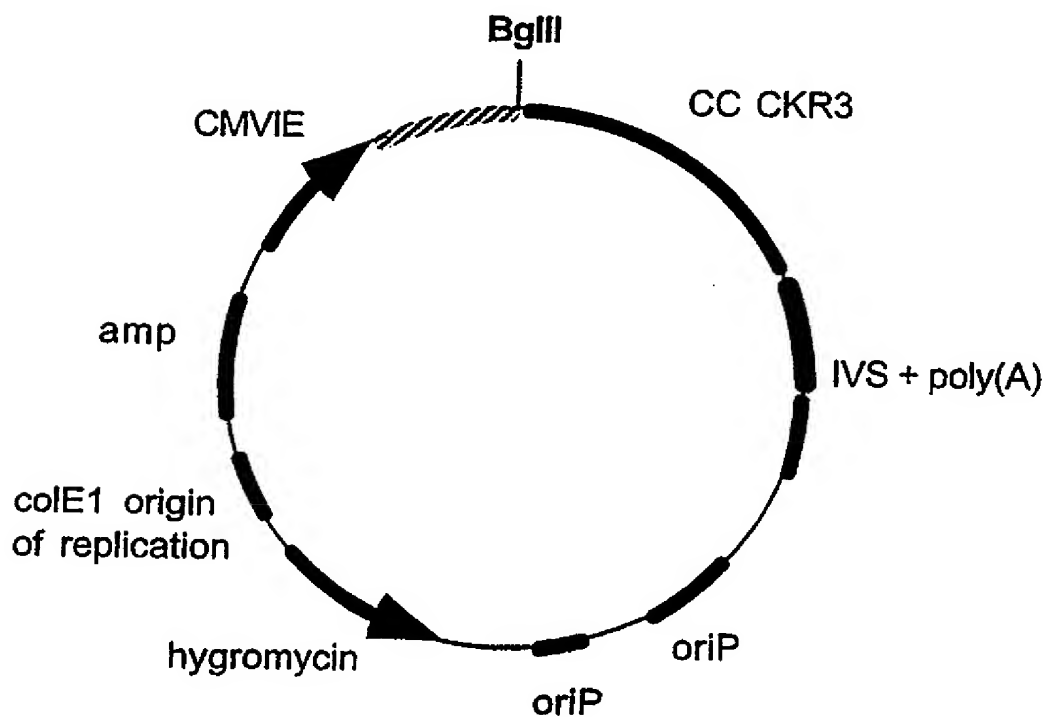
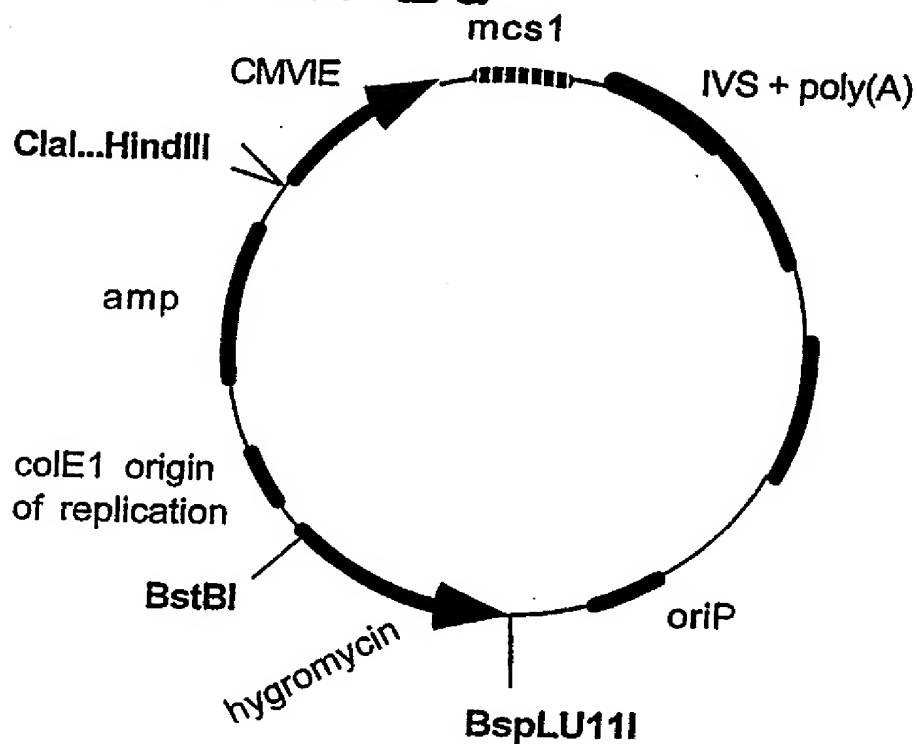
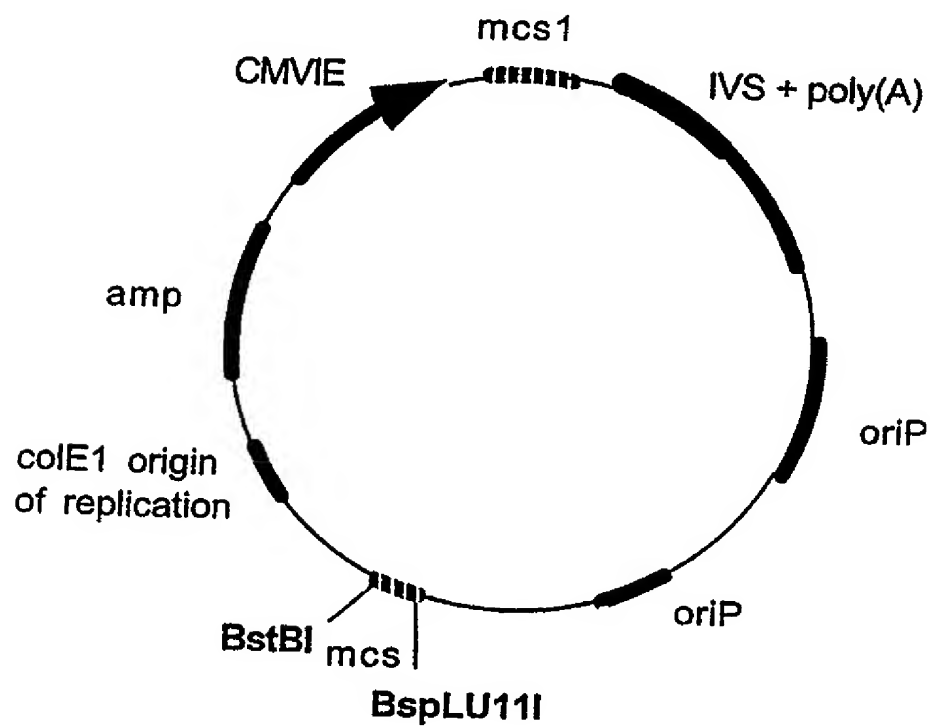


FIG. 1h



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**FIG. 2a****FIG. 2b**

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FIG. 2c

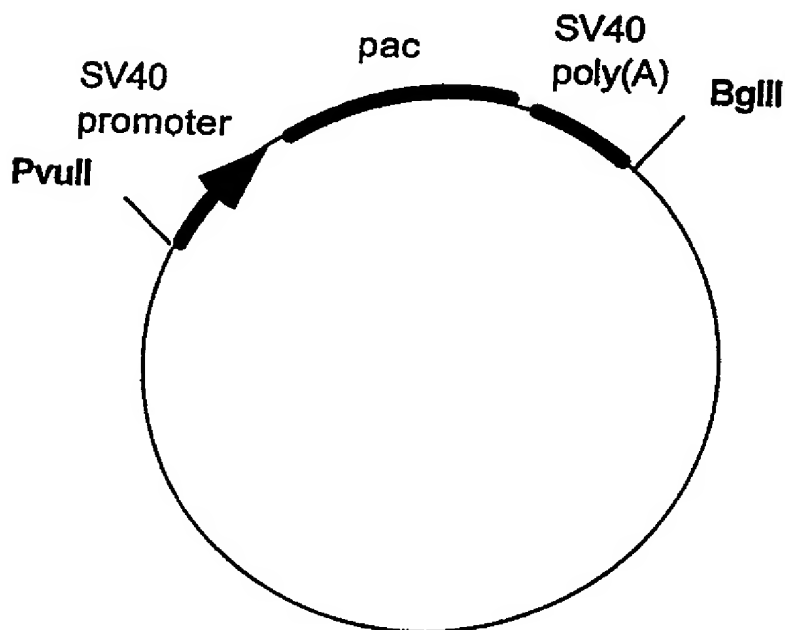
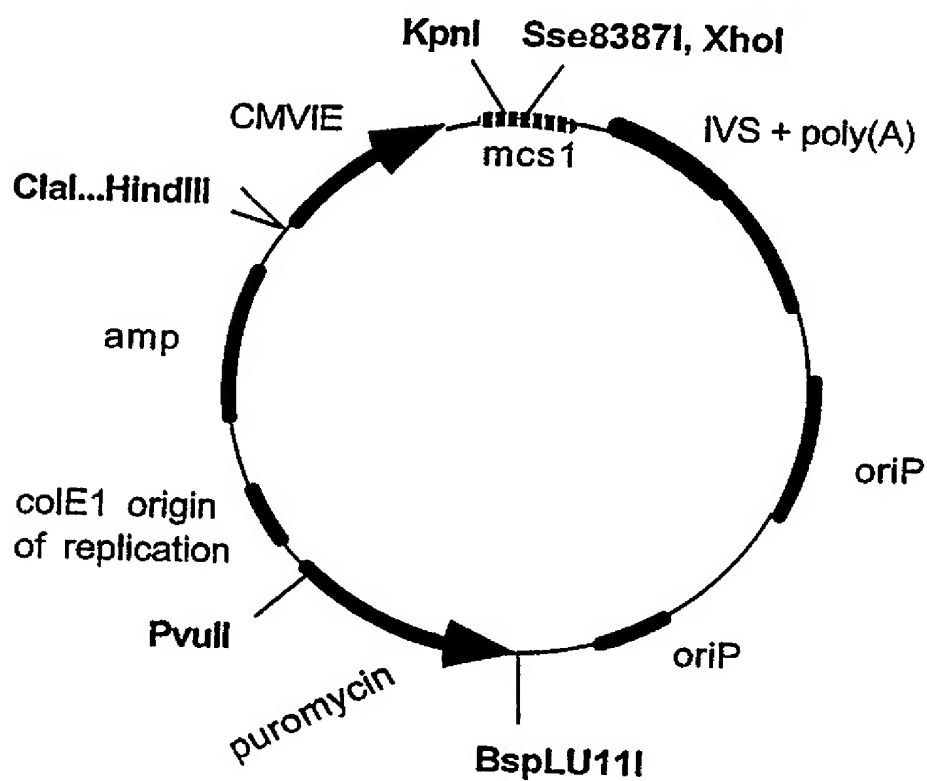


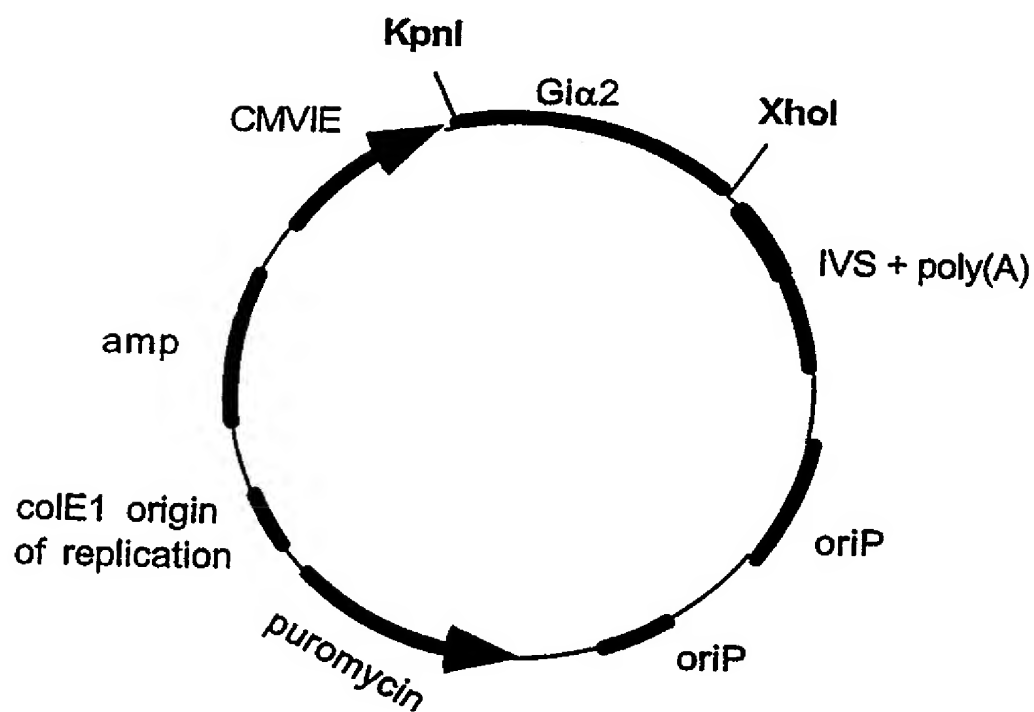
FIG. 2d



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FIG. 2e



## FIG. 3a

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1  TCTAGAGTCG ACCGGTCATG GCTGGGCCCC GACACCCGCC AACACCCGCT
51  GACGCGCCCT GACGGGCTTG TCTGCTCCCG GCATCCGCTT ACAGACAAGC
101  TGTGACCGTC TCCGGGAGCT GCATGTGTCA GAGGTTTCA CCGTCATCAC
151  CGAAACGCGC GAGGCAGCCG GATCATAATC AGCCATACCA CATTGTAGA
201  GGTTTFACTT GCTTTAAAAA ACCTCCCCAC CTCCCCCTGA ACCTGAAACA
251  TAAATGAAT GCAATTGTTG TTGTTAACTT GTTTATTGCA GCTTATAATG
301  GTTACAAATA AAGCAATAGC ATCACAAATT TCACAAATAA AGCATTTT
351  TCACTGCATT CTAGTTGTGG TTTGTCCAAA CTCATCAATG TATCTTATCA
401  TGTCTGGATC CGGCTTGCC GGCCTCGAGC GGCCGCTAGC AAGCTTGCTA
451  GCAGCTGGTA CCCAGCTTCT AGAGATCTGA CGGTTCACTA AACGAGCTCT
501  GCTTATATAG ACCTCCCACC GTACACGCCT ACCGCCCATT TCGGTCAACG
551  GGGCGGGGTT ATTACGACAT TTTGGAAAGT CCCGTTGATT TTGGTGCCAA
601  AACAAACTCC CATTGACGTC AATGGGGTGG AGACTTGGAA ATCCCCGTGA
651  GTCAAACCGC TATCCACGCC CATTGGTGTA CTGCCAAAC CGCATCACCA
701  TGGTAATAGC GATGACTAAT ACGTAGATGT ACTGCCAAGT AGGAAAGTCC
751  CGTAAGGTCA TGTA CTGGG ATAA TGCCAG GCGGGCCATT TACCGTCATT
801  GACGTCAATA GGGGGCGGAC TTGGCATATG ATACACTTGA TGTA CTGCCA
851  AGTGGGCAGT TTACCGTAAA TACTCCACCC ATTGACGTCA ATGGAAGTC
901  CCTATTGGCG TTA CTATGGG AACATACGTC ATTATTGACG TCAATGGGCG
951  GGGTCTGTTG GCGGTCAGC CAGGCGGGCC ATTTACCGTA AGTTATGTAA
1001 CGCGGAATC CATATATGGG CTATGAACTA ATGACCCCGT AATTGATTAC

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A \_\_\_\_\_ A

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## FIG. 3b

A		A	
1051	TATTAATAAC	TAGTCAATAA	TCAATGTCAA
1101	TGAGCCCAATA	TAAATGTACA	TATTATGATA
1151	GCCAATAGCC	AATATTGATT	TATGCTATAT
1201	AATTGCCAAT	ATTGATTCAA	TGTATAGATC
1251	CGCCTGCAGC	AATGCAACAA	CGTTGCCCGG
1301	AAACATGAGA	ATTGGTCGAC	TAGCTTGGCA
1351	GTTTTGGGG	GTCGGGGGTG	TTTGGCAGCC
1401	TCGCGCCAGT	ACATGCGGTC	CATGCCCAGG
1451	TGCTCTGCTCA	GTCAGTCTGT	GGACCAGACC
1501	TAACCCCCAC	GAACCATAAA	CCATTCCCCA
1551	CCACGGGGCC	AGTGGCTATG	GCAGGGCCTG
1601	AGCCCTGGGC	CTTCACCCCGA	ACTTGGGGGG
1651	AACGCGGGCG	TATTGGCCCC	AATGGGGTCT
1701	GCCAGCCCTG	GGACCGAACC	CCGCGTTTAT
1751	GTGCGTTTTA	TTCTGTCTTT	TTATTGCCGT
1801	GTATTGTCTC	CTTCCGTGTT	TCAGTTAGCC
1851	TTTGCCCTCG	GACGAGTGCT	GGGGCGTCGG
1901	TTCTACACAG	CCATCGGTCC	AGACGGCCGC
1951	TACGCCCGAC	AGTCCCGGCT	CCGGATCGGA
2001	TGCGCCCAAG	CTGCATCATC	GAAATTGCCG
2051	TTGGTCAAGA	CCAATGCGGA	GCATATACGC
2101	CAAGCTCCGG	ATGCCCTCCG	TCGAAGTAGC
B		B	
		CATGGCGGTC	ATATTGGACA
		TAGATACAAC	GTATGCAATG
		AACCAATGAC	TAATATGGCT
		TTCCATACCT	ACCAGTTCTG
		ATCTGCGATG	ATAAGCTGTC
		CGCCAGAAAT	CCGCGCGGTG
		ACAGACGCC	GGTGTTCTGT
		CCATCCAAA	ACCATGGGTC
		CCACGCAACG	CCCAAAATAA
		TGGGGGACCC	CGTCCCCTAAC
		CCGCCCCGAC	GTTGGCTGCG
		TGGGGTGGG	AAAGGAAGA
		CGGTGGGTA	TCGACAGAGT
		GAACAAACGA	CCCAACACCC
		CATAGCGCGG	GTTCCCTTCCG
		TCCCCCATCT	CCCCTATTCC
		TTTCCACTAT	CGCGAGTAC
		GCTTCTGCGG	GCGATTGTG
		CGATTGCGTC	GCATCGACCC
		TCAACCAAGC	TCTGATAGAG
		CCGGAGCCGC	GGCGATCCTG
		CGGTCTGCTG	CTCCATACAA

## FIG. 3c

B

2151	GCCAACCACG	GCCTCCAGAA	GAAGATGTTG	GCGACCTCGT	ATTGGGAATC
2201	CCCGAACATC	GCCTCGCTCC	AGTCAATGAC	CGCTGTTATG	CGGCCATTGT
2251	CCGTCAGGAC	ATTGTTGGAG	CCGAAATCCG	CGTGCACGAG	GTGCCGGACT
2301	TCGGGGCAGT	CCTCGGCCCA	AAGCATCAGC	TCATCGAGAG	CCTGCGCGAC
2351	GGACGCACTG	ACGGTGTCGT	CCATCACAGT	TTGCCAGTGA	TACACATGGG
2401	GATCAGCAAT	CGCGCATATG	AAATCACGCC	ATGTAGTGTA	TTGACCGATT
2451	CCTTGCGGTC	CGAATGGGCC	GAACCCGCTC	GTCTGGCTAA	GATCGGCCGC
2501	AGCGATCGCA	TCCATGGCCT	CCGCGACCGG	CTGCAGAAC	GCGGGCAGTT
2551	CGGTTTCAGG	CAGGTCCTGC	AACGTGACAC	CCGTGTCACG	GCGGGAGATG
2601	CAATAGGTCA	GGCTCTCGCT	GAATTCCCCA	ATGTCAAGCA	CTTCCGGAAT
2651	CGGAGCGCG	GCCGATGCAA	AGTGCCGATA	AACATAACGA	TCTTTGTAGA
2701	AACCATCGGC	GCAGCTATT	ACCCGCAGGA	CATATCCACG	CCCTCCTACA
2751	TCGAAGCTGA	AAGCACGAGA	TTCTTCGCCC	TCCGAGAGCT	GCATCAGGTC
2801	GGAGACGCTG	TCGAACTTTT	CGATCAGAAA	CTTCTCGACA	GACGTCGCGG
2851	TGAGTTCAGG	CTTTTTCATA	TCTCATTGCC	CGGGATCTGC	GGCACGCTGT
2901	TGACGCTGTT	AAGCGGGTCG	CTGCAGGGTC	GCTCGGTGTT	CGAGGCCACA
2951	CGCGTCACCT	TAATATGCGA	AGTGGACCTG	GGACCGCGCC	GCCCCGACTG
3001	CATCTGCGTG	TTTCGAATTTCG	CCAATGACAA	GACGCTGGGC	GGGGTTTGTG
3051	TCATCATAGA	ACTAAAGACA	TGCAAAATATA	TTTCTTCCGG	GGACACCGCC
3101	AGCAAACGCG	AGCAAACGGC	CACGGGGATG	AAGCAGGGCA	TGGCGGCCGA
3151	CGCGCTGGGC	TACGTCTTGC	TGGCGTTCGC	GACGCGAGGC	TGGATGGCCT

C

**FIG. 3d**

C  
 3201 TCCCCATTAT GATTCTTCTC GCTTCCGGCG GCATCGGGAT GCCCGCGTTG  
 3251 CAGGCCATGC TGTCCAGGCA GGTAGATGAC GACCATCAGG GACAGCTTCA  
 3301 AGGATCGCTC GCGGCTCTTA CCAGCCTAAC TTCGATCACT GGACCGCTGA  
 3351 TCGTCACGGC GATTTA TGCC GCCTCGGCGA GCACATGGAA CGGGTTGGCA  
 3401 TGGATTGTAG GCGCGCCCT ATACCTTGTC TGCCTCCCG CGTTGCGTCG  
 3451 CGGTGCATGG AGCCGGGCCA CCTCGACCTG AATGGAAGCC GCGGCACCT  
 3501 CGCTAACGGA TTCACCACTC CAAGAATTGG AGCCAATCAA TTCTTGCGGA  
 3551 GAACTGTGAA TGCGCAAACC AACCTTGGC AGAACATATC CATCGCGTCC  
 3601 GCCATCTCCA GCAGCCGCAC GCGGCGCAGC AAAAGGCCAG GAACCGTAAA  
 3651 AAGGCCGCGT TGCTGGCGTT TTTC CATAGG CTCCGCCCCC CTGACGAGCA  
 3701 TCACAAAAAT CGACGCTCAA GTCAGAGGTG GCGAAACCCG ACAGGACTAT  
 3751 AAAGATACCA GCGGTTTCCC CCTGGAAGCT CCCTCGTGCG CTCTCCTGTT  
 3801 CCGACCCCTGC CGCTTACCGG ATACCTGTCC GCCTTTCTCC CTTCCGGGAAG  
 3851 CGTGGCGCTT TCTCATAGCT CACGCTGTAG GTATCTCAGT TCGGTGTAGG  
 3901 TCGTTTCGCTC CAAGCTGGGC TGTGTGCACG AACCCCCCGT TCAGCCCGAC  
 3951 CGCTGCGCCT TATCCGGTAA CTATCGTCTT GAGTCCAACC CGGTAAGACA  
 4001 CGACTTATCG CCACTGGCAG CAGCCACTGG TAACAGGATT AGCAGAGCGA  
 4051 GGATGTAGG CCGTGCTACA GAGTTCTTGA AGTGGTGGCC TAACTACGGC  
 4101 TACACTAGAA GGACAGTATT TGGTATCTGC GCTCTGCTGA AGCCAGTTAC  
 4151 CTTCGGAAAA AGAGTTGGTA GCTCTTGATC CGGCAAACAA ACCACCGCTG  
 4201 GTAGCGGTGG TTTT TTTTGT TGC AAGCAGC AGATTACGCG CAGAAAAAAA  
 4251 GGATCTCAAG AAGATCCTTT GATCTTTTCT ACGGGGTCTG ACGCTCAGTG  
 D

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## FIG. 3e

D

4301 GAACGAAAC TCACGTTAAG GGATTTTGGT CATGAGATTA TCAAAAGGA  
4351 TCTTCACCTA GATCCTTTTA AATTAAAAAT GAAGTTTAA ATCAATCTAA

4401 AGTATATATG AGTAAACTTG GTCTGACAGT TACCAATGCT TAATCAGTGA  
4451 GGCACCTATC TCAGCGATCT GTCTATTTCG TTCATCCATA GTTGCCCTGAC  
4501 TCCCCGTCGT GTAGATAACT ACGATACGGG AGGCTTACC ATCTGGCCCC  
4551 AGTGCTGCAA TGATACCGCG AGACCCACGC TCACCCGGTC CAGATTATC  
4601 AGCAATAAAC CAGCCAGCCG GAAGGGCCGA GCGCAGAACT GGTCTGCAA  
4651 CTTTATCCGC CTCATCCAG TCTATTAAAT GTTGCCGGA AGCTAGAGTA  
4701 AGTAGTTTCG CAGTTAATAG TTTGCGCAAC GTTGTGCGA TTGCTGCAAG  
4751 CATCGTGGTG TCACGCTCGT CGTTTGGTAT GGCTTCATTC AGCTCCGGTT  
4801 CCCAACGATC AAGCGAGTT ACATGATCCC CCATGTTGTG CAAAAAGCG  
4851 GTTAGCTCCT TCGGTCCTCC GATCGTTGTC AGAAGTAAGT TGGCCGCAGT  
4901 GTTATCACTC ATGGTTATGG CAGCACTGCA TAATTCTCTT ACTGTCATGC  
4951 CATCCGTAAG ATGCTTTTCT GTGACTGGTG AGTACTCAAC CAAGTCATTG  
5001 TGAGAAATAG GTATGCGGCG ACCGAGTTGC TCTTGCCCCG CGTCAACACG  
5051 GGATAATACC GCGCCACATA GCAGAACTTT AAAAGTGCTC ATCATTTGAA  
5101 AACGTTCTTC GGGGCGAAAA CTCTCAAGGA TCTTACCGCT GTTGAGATCC  
5151 AGTTCGATGT AACCCACTCG TGCACCCAAC TGATCTTCAG CATCTTTTAC  
5201 TTTCAACCAGC GTTCTCTGGGT GAGCAAAAAC AGGAAGGCAA AATGCCGCAA

E

5251	AAAAGGGAAT	AAGGGCGACA	CGAAATGTT	GAATACTCAT	ACTCTTCCTT
5301	TTTCAATATT	ATTGAAGCAT	TTATCAGGGT	TATTGTCTCA	TGAGCGGATA
5351	CATATTTGAA	TGTATTTAGA	AAAATAAACA	AATAGGGTT	CCGCGCACAT
5401	TTCCCCGAAA	AGTGCCACCT	GACGTCTAAG	AAACCATTAT	TATCATGACA
5451	TTAACCTATA	AAAATAGGCG	TATCACGAGG	CCCTTTCTGC	TTCAAGAATT
5501	CTCATGTTG	ACAGCTTATC	ATCGATAAGC	TGATCCTCAC	AGGCCGCACC
5551	CAGCTTTTCT	TCCGTTGCCC	CAGTAGCATC	TCTGTCTGGT	GACCTTGAAG
5601	AGGAAGAGGA	GGGTCCCGA	GAATCCCCAT	CCCTACCGTC	CAGCAAAAAG
5651	GGGACGAGG	AATTGAGGC	CTGGCTTGAG	GCTCAGGACG	CAAATCTTGA
5701	GGATGTTTTCAG	CGGAGTTT	CCGGGCTGCG	AGTAATTGGT	GATGAGGACG
5751	AGGATGGTTC	GGAGGATGGG	GAATTTTCAG	ACCTGGATCT	GTCTGACAGC
5801	GACCATGAAG	GGGATGAGGG	TGGGGGGGCT	GTTGGAGGGG	GCAGGAGTCT
5851	GCACTCCCTG	TATTCACCTGA	GCGTCGTCTA	ATAAAGATGT	CTATTGATCT
5901	CTTTTAGTGT	GAATCATGTC	TGACGAGGGG	CCAGGTACAG	GACCTGGAAG
5951	TGGCCTTAGGA	GAGAAAGGAG	ACACATCTGG	ACCAGAAGGC	TCCGGCGGCA
6001	GTGGACCTCA	AAGAAGAGGG	GGTGATAACC	ATGACGAGG	ACGGGGAAGA
6051	GGACGAGGAC	GAGGAGGCGG	AAGACCAGGA	GCCCCGGCGG	GCTCAGGATC
6101	AGGGCCAAGA	CATAGAGATG	GTGTCCGGAG	ACCCCAAAA	CGTCCAAGTT
6151	GCATTGGCTG	CAAAGGGACC	CACGGTGGAA	CAGGAGCAGG	AGCAGGAGCG
6201	GGAGGGGCAG	GAGCAGGAGG	GGCAGGAGCA	GGAGGAGGGG	CAGGAGCAGG
6251	AGGAGGGGCA	GGAGGGGCAG	GAGGGGCAGG	AGGGGCAGGA	GCAGGAGGAG
6301	GGGCAGGAGC	AGGAGGAGGG	GCAGGAGGGG	CAGGAGGGGC	AGGAGCAGGA
6351	GGAGGGGCAG	GAGCAGGAGG	AGGGGCAGGA	GGGCAGGAG	CAGGAGGAGG



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## FIG. 3g

F \_\_\_\_\_

6401	GGCAGGAGGG	GCAGGAGGGG	CAGGAGCAGG	AGGAGGGGCA	GGAGCAGGAG
6451	GAGGGGCAGG	AGGGGCAGGA	GCAGGAGGAG	GGGCAGGAGG	GGCAGGAGGG
6501	GCAGGAGCAG	GAGGAGGGGC	AGGAGCAGGA	GGGCAGGAG	GGGCAGGAGG
6551	GGCAGGAGCA	GGAGGGGCAG	GAGCAGGAGG	AGGGGCAGGA	GGGCAGGAG
6601	GGCAGGAGC	AGGAGGGGCA	GGAGCAGGAG	GGGCAGGAGC	AGGAGGGGCA
6651	GGAGCAGGAG	GGGCAGGAGG	GGCAGGAGCA	GGAGGGGCAG	GAGGGGCAGG
6701	AGCAGGAGGG	GCAGGAGGGG	CAGGAGCAGG	AGGAGGGGCA	GGAGGGGCAG
6751	GAGCAGGAGG	AGGGGCAGGA	GGGCAGGAG	CAGGAGGGC	AGGAGGGGCA
6801	GGAGCAGGAG	GGGCAGGAGG	GGCAGGAGCA	GGAGGGGCAG	GAGGGGCAGG
6851	AGCAGGAGGA	GGGCAGGAG	CAGGAGGGC	AGGAGCAGGA	GGTGAGGGCC
6901	GGGTTCGAGG	AGGCAGTGGA	GGCCGGGGTC	GAGGAGGTAG	TGGAGGGCCGG
6951	GGTCGAGGAG	GTAGTGAGG	CCGCCGGGGT	AGAGGACGTG	AAAGAGCCAG
7001	GGGGGGAAGT	CGTGAAAGAG	CCAGGGGGAG	AGGTCGTGGA	CGTGGAGAAA
7051	AGAGGGCCAG	GAGTCCCAGT	AGTCAGTCAT	CATCATCCGG	GTCTCCACCG
7101	CGCAGGCCCC	CTCCAGGTAG	AAGCCATT	TTCCACCCCTG	TAGGGGAAGC
7151	CGATTATT	GAATACCACC	AAGAAGGTGG	CCCAGATGGT	GAGCCTGACG
7201	TGCCCCCGGG	AGCGATAGAG	CAGGGCCCCG	CAGATGACCC	AGGAGAAGGC
7251	CCAAGCACTG	GACCCCGGGG	TCAGGGTGAT	GGAGGCAGGC	GCAAAAAGG
7301	AGGGTGTTT	GGAAAGCATC	GTGGTCAAGG	AGGTTCCAAC	CCGAAATTG
7351	AGAACATTGC	AGAAGGTTA	AGAGCTCTCC	TGGCTAGGAG	TCACGTAGAA
7401	AGGACTACCG	ACGAAGGAAC	TTGGGTCGCC	GGTGTGTTCC	TATATGGAGG
7451	TAGTAAGACC	TCCCTTTACA	ACCTAAGGCG	AGGAACTGCC	CTTGCTATTC

\_\_\_\_\_ G

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## FIG. 3h

G										H									
7501	CACAATGTCG	TCTTACACCA	TTGAGTCGTC	TCCCCCTTGG	AATGGCCCCT														
7551	GGACCCGGCC	CACAACCTGG	CCCGCTAAGG	GAGTCCATTG	TCTGTATTAT														
7601	CATGGTCTTT	TTACAAACTC	ATATATTGTC	TGAGGTTTGG	AAGGATGCCA														
7651	TTAAGGACCT	TGTTATGACA	AAGCCCGCTC	CTACCTGCAA	TATCAGGGTG														
7701	ACTGTGTGCA	GCTTTGACGA	TGGAGTAGAT	TTGCCCTCCCT	GTTTCCACC														
7751	TATGTTGGAA	GGGCTGCCG	CGGAGGTGA	TGACGGAGAT	GACGGAGATG														
7801	AAGGAGGTGA	TGGAGATGAG	GGTGAGGAAG	GGCAGGAGTG	ATGTAACTTG														
7851	TTAGGAGACG	CCCTCAATCG	TATTAAAAGC	CGTGTATTCC	CCCGCACTAA														
7901	AGAAATAAATC	CCCAGTAGAC	ATCATGCGTG	CTGTTGGTGT	ATTCTGGCC														
7951	ATCTGTCTTG	TCACCATTTT	CGTCCTCCCA	ACATGGGCA	ATTGGGCATA														
8001	CCCATGTTGT	CACGTCACCTC	AGCTCCGCGC	TCAACACCTT	CTCGCGTTGG														
8051	AAAACATTAG	CGACATTTAC	CTGGTGAGCA	ATCAGACATG	CGACGGCTTT														
8101	AGCCTGGCCT	CCTTAAATTC	ACCTAAGAAAT	GGGAGCAACC	AGCATGCAGG														
8151	AAAAGGACAA	GCAGCGAAAA	TTTACCGCCCC	CTTGGGAGGT	GGCGGCATAT														
8201	GCAAAGGATA	GCACTCCAC	TCTACTACTG	GGTATCATAT	GCTGACTGTA														
8251	TATGCATGAG	GATAGCATAT	GCTACCCGGA	TACAGATTAG	GATAGCATAT														
8301	ACTACCCAGA	TATAGATTAG	GATAGCATAT	GCTACCCAGA	TATAGATTAG														
8351	GATAGCCTAT	GCTACCCAGA	TATAAATTAG	GATAGCATAT	ACTACCCAGA														
8401	TATAGATTAG	GATAGCATAT	GCTACCCAGA	TATAGATTAG	GATAGCCTAT														
8451	GCTACCCAGA	TATAGATTAG	GATAGCATAT	GCTACCCAGA	TATAGATTAG														
8501	GATAGCATAT	GCTATCCAGA	TATTGGGTA	GTATATGCTA	CCCAGATATA														

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H		H
8551	AATTAGGATA GCATATACTA CCCTAATCTC TATTAGGATA GCATATGCTA	
8601	CCCGGATACA GATTAGGATA GCATATACTA CCCAGATATA GATTAGGATA	
8651	GCATATGCTA CCCAGATATA GATTAGGATA GCCTATGCTA CCCAGATATA	
8701	AATTAGGATA GCATATACTA CCCAGATATA GATTAGGATA GCATATGCTA	
8751	CCCAGATATA GATTAGGATA GCCTATGCTA CCCAGATATA GATTAGGATA	
8801	GCATATGCTA TCCAGATATT TGGGTAGTAT ATGCTACCCA TGGCAACATT	
8851	AGCCCCACCGT GCTCTCAGCG ACCTCGTGAA TATGAGGACC AACAAACCCCTG	
8901	TGCTTTGGCG TCAGGCGCAA GTGTGTGTAA TTTGTCTCTCC AGATCGCAGC	
8951	AATCGCGCCC CTATCTTGGC CCGCCCCACCT ACTTATGCAG GTATTCCCCG	
9001	GGGTGCCCATT AGTGGTTTTG TGGGCAAGTG GTTTGACCGC AGTGGTTAGC	
9051	GGGGTTACAA TCAGCCAAGT TATTACACCC TTATTTTACA GTCCAAAACC	
9101	GCAGGGCGGC GTGTGGGGC TGACGCGTGC CCCACTCCA CAATTTCAAA	
9151	AAAAAGAGTG GCCACTTGTC TTTGTTTATG GGCCCCATTG GCGTGGAGCC	
9201	CCGTTTAATT TTCGGGGGTG TTAGAGACAA CCAGTGGAGT CCGCTGCTGT	
9251	CGGCGTCCAC TCTCTTTCCC CTTGTTACAA ATAGAGTGTA ACAACATGGT	
9301	TCACCTGTCT TGGTCCCTGC CTGGGACACA TCTTAATAAC CCCAGTATCA	
9351	TATTGCACTA GGATTATGTG TTGCCCATAG CCATAAATTC GTGTGAGATG	
9401	GACATCCAGT CTTTACGGCT TGTCCCCACC CCATGGATTI CTATTGTTAA	
9451	AGATATTCAG AATGTTTCAT TCCTACACTA GTATTTATTG CCCAAGGGGT	I

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## FIG. 3j

I

9501	TTGTGAGGGT	TATATTGGTG	TCATAGACA	ATGCCACCAC	TGAACCCCCC
9551	GTCCAAATTT	TATTCTGGGG	GCGTCACCTG	AAACCTTGTT	TTCGAGCACC
9601	TCACATACAC	CTTACTGTTC	ACAACTCAGC	AGTTATTCTA	TTAGCTAAAC
9651	GAAGGAGAAT	GAAGAAGCAG	GCGAAGATTC	AGGAGAGTTC	ACTGCCCGCT
9701	CCTTGATCTT	CAGCCACTGC	CCTTGTGACT	AAAATGGTTC	ACTACCCCTCG
9751	TGGAATCCCTG	ACCCCATGTA	AATAAAACCG	TGACAGCTCA	TGGGGTGGGA
9801	GATATCGCTG	TTCCCTTAGGA	CCCTTTTACT	AACCCTAATT	CGATAGCATA
9851	TGCTTCCCGT	TGGGTAACAT	ATGCTATTGA	ATTAGGGTTA	GTCTGGATAG
9901	TATATACTAC	TACCCGGGAA	GCATATGCTA	CCC GTTTAGG	GTTAACAAGG
9951	GGCCTTATA	AACACTATTG	CTAATGCCCT	CTTGAGGGTC	CGCTTATCGG
10001	TAGCTACACA	GGCCCCCTCTG	ATTGACGTTG	GTGTAGCCTC	CCGTAGTCTT
10051	CCTGGGCCCC	TGGGAGGTAC	ATGTCCCCCA	GCATTGGTGT	AAGAGCTTCA
10101	GCCAAGAGTT	ACACATAAAG	GCAATGTTGT	GTTGCAGTCC	ACAGACTGCA
10151	AAGTCTGCTC	CAGGATGAAA	GCCACTCAGT	GTTGGCAAAT	GTGCACATCC
10201	ATTTATAAGG	ATGTCAACTA	CAGTCAGAGA	ACCCCTTTGT	GTTTGGTGCC
10251	CCCCCGTGTC	ACATGTGGAA	CAGGGCCCAG	TTGGCAAGTT	GTACCAACCA
10301	ACTGAAGGGA	TTACATGCAC	TGCCCCCGCA	AGAAGGGCA	GAGATGCCGT
10351	AGTCAGGTTT	AGTTCGTCCG	GCGCGGGGC		

J

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## FIG. 4a

```

1  GCGGTAATCT GCTGCTTGCA AACAAAAAAA CCACCGCTAC CAGCGGTGGT
51  TTGTTTGCCG GATCAAGAGC TACCAACTCT TTTTCCGAAG GTAAC TGGCT
101 TCAGCAGAGC GCAGATACCA AATACTGTCC TTCTAGTGA GCCGTAGTTA
151 GGCCACCACCT TCAAGAACTC TGTAGCACCG CCTACATACC TCGCTCTGCT
201 AATCCTGTTA CCAGTGGCTG CTGCCAGTGG CGATAAGTCG TGTCTTACCG
251 GGTGGAATC AAGACGATAG TTACCCGGATA AGGCGCAGCG GTCGGGCTGA
301 ACGGGGGGTT CGTGACACAGA GCCCAGCTTG GAGCGAACGA CCTACACCGA
351 ACTGAGATAC CTACAGCGTG AGCATTGAGA AAGCGCCACG CTTCCCGAAG
401 GGAGAAAGGC GGACAGGTAT CCGGTAAGCG GCAGGGTCGG AACAGGAGAG
451 CGCACGAGGG AGCTTCCAGG GGGAAACGCC TGCTATCTTT ATGATCCTGT
501 CGGGTTTCGC CACCTCTGAC TTGAGCGTCG ATTTTGTGA TGCTCGTCAG
551 GGGGGCGGAG CCTATGGAAA AACGCCAGCA ACGCAAGCTA GCTTCTAGCT
601 AGAAATTGTA AACGTTAATA TTTTGTAA ATTCGCGTTA AATTTTGT
651 AAATCAGCTC ATTTTTTAA CAATAGGCCG AAATCGGCAA AATCCCTTAT
701 AAATCAAAAG AATAGCCCCG GATAGGGTTG AGTGTGTTTC CAGTTTGAA
751 CAAGAGTCCA CTATTAAAGA ACGTGACTC CAACGTCAA GGGCGAAAAA
801 CCGTCTATCA GGGCGATGGC CGCCCACTAC GTGAACCATC ACCCAAATCA
851 AGTTTTTGTG GGTGAGGTG CCGTAAAGCA CTAAATCGGA ACCCTAAAGG
901 GAGCCCCCGA TTTAGAGCTT GACGGGGAAA GCCGGCGAAC GTGGCGAGAA
951 AGGAAGGGAA GAAAGCGAAA GGAGCGGGCG CTAGGGCGCT GGCAAGTGA
1001 GCGGTACGC TCGCGGTAAC CACCACACCC GCCGCGCTTA ATGCGCCGT
1051 ACAGGGCGCG TACTATGGTT GCTTTGACGA GACCGTATAA CGTGCTTTCC
A

```

## FIG. 4b

A	A
1101	TCGTTGGAAT CAGAGCGGA GCTAAACAGG AGGCCGATTA AAGGATTTT
1151	AGACAGGAAC GGTACGCCAG CTGGATTACC GCGTCTTTC TCAACGTAAC
1201	ACTTTACAGC GCGCGTTCAT TTGATATGAT GCGCCCGCT TCCCGATAAG
1251	GGAGCAGGCC AGTAAAGCA TTACCCGTGG TGGGTTCCT GAGCGGCCAA
1301	AGGAGGCAGA CTCTAAATCT GCCGTCAATG ACTTCGAAGG TTCGAATCCT
1351	TCCCCCACCA CCATCACTTT CAAAGTCCG AAAGAATCTG CTCCTGCTT
1401	GTGTGTTGGA GGTGCTGAG TAGTGCGCGA GTAAAATTTA AGCTACAACA
1451	AGGCAAGGCT TGACCGACAA TTGCATGAAG AATCTGCTTA GGGTTAGCG
1501	TTTTGCGCTG CTTTCGCGATG TACGGGCCAG ATATACGCGT TGACATTGAT
1551	TATTGACTAG TTATTAAATG TAATCAATTA CGGGTCATT AGTTCATAGC
1601	CCATATATGG AGTTCCGCGT TACATAACTT ACGGTAAATG GCCCGCCTGG
1651	CTGACCGCCC AACGACCCC GCCCATTGAC GTCAATAATG ACGTATGTC
1701	CCATAGTAAC GCCAATAGGG ACTTTCCATT GACGTCAATG GGTGACTAT
1751	TTACGGTAAA CTGCCCCTT GGCAGTACAT CAAGTGATC ATATGCCAAG
1801	TACGCCCCCT ATTGACGTCA ATGACGGTAA ATGGCCCGC TGGCATATG
1851	CCCAGTACAT GACCTTATGG GACTTTCCTA CTTGGCAGTA CATCTACGTA
1901	TTAGTCAATG CTATTACCAT GGTATGCGG TTTTGGCAGT ACATCAATGG
1951	GCGTGGATAG CGGTTTGACT CACGGGGATT TCCAAGTCTC CACCCCATG
2001	ACGTCAATGG GAGTTTGTTT TGGCACCAA ATCAACGGA CTTTCCAAA
2051	TGTCGTAACA ACTCCGCCCC ATTGACGCAA ATGGGCGGTA GCGGTGTACG
2101	GTGGGAGGTC TATATAAGCA GAGCTCTCTG GCTAACTAGA GAACCCACTG
B	B

## FIG. 4c

B	2151	CTTACTGGCT	TATCGAAATT	AATACGACTC	ACTATAGGGA	GACCCAAAGCT	B
	2201	TCTAGAGATC	CCTCGACCTC	GAGATCCATT	GTGCTGGCGC	GGATTCTTTA	
	2251	TCACTGATAA	GTTGGTGGAC	ATATFATGTT	TATCAGTGAT	AAAGTGTCAA	
	2301	GCATGACAAA	GTTGCAGCCG	AATACAGTGA	TCCGTGCCGG	CCCTGGACTG	
	2351	TTGAAACGAG	TCGGCGTAGA	CGTCTGACG	ACACGCAAC	TGGCGGAACG	
	2401	GTTGGGGTG	CAGCAGCCGG	CGCTTTACTG	GCACTTCAGG	AACAAGCGGG	
	2451	CGCTGCTCGA	CGCACTGGCC	GAAGCCATGC	TGGCGGAGAA	TCATACGCTT	
	2501	CGGTGCCGAG	AGCCGACGAC	GACTGGCGCT	CATTTCTGAT	CGGGAATCCC	
	2551	GCAGCTTCAG	GCAGGCGCTG	CTCGCCTACC	GCCAGCACAA	TGGATCTCGA	
	2601	GGGATCTTCC	ATACCTACCA	GTTCTGCGCC	TGCAGGTCGC	GGCCGCGACT	
	2651	CTAGAGGATC	TTTGTGAAGG	AACCTTACTT	CTGTGGTGTG	ACATAAATTGG	
	2701	ACAAACTACC	TACAGAGATT	TAAAGCTCTA	AGGTAATAAT	AAAATTTTTA	
	2751	AGTGTATAAT	GTGTTAAACT	ACTGATTCTA	ATTGTTGTGG	TATTTAGAT	
	2801	TCCAACCTAT	GGAACCTATG	AATGGGAGCA	GTGGTGGAAT	GCCTTTAATG	
	2851	AGGAAACCT	GTTTGTGCTCA	GAAGAAATGC	CATCTAGTGA	TGATGAGGCT	
	2901	ACTGCTGACT	CTCAACATTC	TACTCCTCCA	AAAAAGAAGA	GAAAGGTAGA	
	2951	AGACCCCAAG	GACTTTCCTT	CAGAATTGGT	AAGTTTTTGG	AGTCATGCTG	
	3001	TGTTTAGTAA	TAGAACTCTT	GCTGCTTTG	CTATTTACAC	CACAAAGGAA	
	3051	AAAGCTGCAC	TGCTATACAA	GAAAATTATG	GAAAAATATT	TGATGTATAG	
	3101	TGCCCTTGACT	AGAGATCATA	ATCAGCCATA	CCACATTTGT	AGAGGTTTTA	
	3151	CTTGCTTTAA	AAAACCTCCC	ACACCTCCCC	CTGAACCTGA	AACATAAAAT	
	3201	GAATGCAATT	GTTGTTGTTA	ACTTGTTTAT	TGCAGCTTAT	AATGGTTACA	C

## FIG. 4d

C

3251	AATAAAGCAA	TAGCATCACA	AATTTCACAA	ATAAAGCATT	TTTATCACTG
3301	CATTCTAGTT	GTGGTTTGTC	CAAACATC	AATGTATCTT	ATCATGTCTG
3351	GATCCCGCCA	TGGTATCAAC	GCCATATTTC	TATTTACAGT	AGGGACCTCT
3401	TCGTTGTGTA	GGTACCGCTG	TATTCCTPAG	GAAATAGTAG	AGGCACCTTG
3451	AACTGTCTGC	ATCAGCCATA	TAGCCCCGC	TGTTGACTT	ACAAACACAG
3501	GCACAGTACT	GACAAACCCA	TACACCTCCT	CTGAAATACC	CATAGTTGCT
3551	AGGGCTGTCT	CCGAACTCAT	TACACCTTAC	CAAGTGAGAG	CTGTAATTTC
3601	GCGATCAAGG	GCAGCGAGGG	CTTCTCCAGA	TAAATAGCT	TCTGCCGAGA
3651	GTCCCGTAAG	GGTAGACACT	TCAGCTAATC	CCTCGATGAG	GTCTACTAGA
3701	ATAGTCAGTG	CGGCTCCCAT	TTTGAAAATT	CACTTACTTG	ATCAGCTTCA
3751	GAAGATGGGC	GAGGGCTCTC	AACACAGTAA	TTTTTCCTCC	GACTCTTAAA
3801	ATAGAAAATG	TCAAGTCACT	TAAGGAGGAA	GTGGACTAAC	TGACGCAGCT
3851	GGCCGTGCGA	CATCCTCTTT	TAATTAGTTG	CTAGGCAACG	CCCTCCAGAG
3901	GGCGTGTGGT	TTTGCAAGAG	GAAGCAAAAG	CCCTCTCCACC	CAGGCCCTAGA
3951	ATGTTTCCAC	CCAATCATTA	CTATGACAAC	AGCTGTTTTT	TTTAGTATTA
4001	AGCAGAGGCC	GGGACCCCT	GGGCCCCGCTT	ACTCTGGAGA	AAAGAAGAG
4051	AGGCATTGTA	GAGGCTTCCA	GAGGCAACTT	GTCAAAACAG	GACTGCTTCT
4101	ATTTCTGTCA	CACGTGCTGG	CCCTGTCAAC	AGGTCCAGCA	CCTCCATACC
4151	CCCTTTAATA	AGCAGTTTGG	GAACGGGTGC	GGTCTTACT	CCGCCCATCC
4201	CGCCCCTAAC	TCCGCCCAGT	TCCGCCCATT	CTCCGCCCCA	TGGCTGACTA
4251	ATTTTTTTTA	TTTATGCAGA	GGCCGAGGCC	GCCTCGGCCT	CTGAGCTATT
4301	CCAGAAAGTAG	TGAGGAGGCT	TTTTTTGGAGG	CCTAGGCTTT	TGCAAAAAGC
4351	TAATTC				



## FIG. 5a

CACCTAAATTGTAAGCGTTAATAATTTGTGTTAAATTCGCGTTAAATTTTGTAAATCAGCTCATTTTAAACCA  
ATAGGCCGAAATCGGCAAAATCCCTTATAAATCAAAAGAAATAGACCGAGATAGGTTGAGTGTGTTCCAGTTTG  
GAACAAGAGTCCACTATTAAAGAACGTGACTCCAACGTCAAAGGGCGAAACCCGTCTATCAGGGCGATGGCCC  
ACTACGTGAACCATCACCCCTAATCAAGTTTGTGGGTCGAGGTGCCGTAAAGCACATAATCGGAACCTTAAAGG  
GAGCCCCGATTTAGAGCTTGACGGGAAAGCCGGCAACGTGGCGAGAAAGGAAGAAAGCGAAAGGAGC  
GGCGCTAGGGCGCTGGCAAGTGTAGCGTCAAGTGCCTGCGGTAAACCAACACCCGCCGCTTAATGCGCCGT  
ACAGGCGGTCCCATTCGCCATTCAGGCTGCGCAACTGTGGGAAGGCGATCGGTGCGGCCCTTTCGCTATT  
ACGCCAGCTGGCGAAAGGGGATGTCTGCAAGCGATTAAAGTTGGGTAAACGCCAGGTTTCCCAGTCACGACG  
TTGTAAACGACGGCCAGTGAATTGTAATACGACTCACTATAGGGCGAATTGGGTACCGGGCCCCCTCGAGGT  
CGACGGTATCGATAAGCTTGATAATCGAATTCCTGCAGCCCGGGGATCCACTAGTCTAGAGCGGCCGCCACCGC  
GGTGAGCTCCAGCTTTTGTTCCTTTAGTGAGGTTAATTCGAGCTTGCGTAATCATGGTCATAGCTGTTTC  
CTGTGTGAAATTGTTATCCGCTCACAAATCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCTGGGGTG  
CCTAATGAGTGAGCTAACTCACATTAAATTGCGTTGCGTCACTGCCCGCTTTCAGTCGGGAAACCTGTCGTGCC  
AGCTGCATTAAATCGGCCAACGCGCGGGAGAGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCA  
CTGACTCGCTCGGTCGTTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGCGGTAATACGGTTATCCA  
CAGAAATCAGGGGATAACGAGGAAAGAACATGTAGCAAAAGGCCAGCAAAAGCCAGGAACCGTAAAGGCCG  
CGTTGCTGGCGTTTTCATAGGCTCCGCCCTGACGAGCATCAAAAATCGACGCTCAAGTCAGAGGTGGC  
GAAACCCGACAGGACTATAAGATACAGGCGTTTCCCTGGAAGCTCCCTCGTGGCTCTCCTGTTCGACCC  
TGCCGCTTACCGGATACCTGTCCGCCCTTTCCTCCCTTCGGGAAGCGTGGCGCTTCTCTCATAGCTCAGCTGTAGGT  
ATCTCAGTTCGGTGTAGTCTGCTCCAGCTGGGCTGTGTGCACGAACCCCGCTTCAGCCGACCGCTGCG

A

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## FIG. 5b

A \_\_\_\_\_ A

CCTTATCCGGTAACTATCGTCTTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTA  
ACAGGATTAGCAGAGCGAGGTATGTAGCGGTGCTACAGAGTTCTTGAAAGTGGTGGCCTAACTACGGCTACACTA  
GAAGACAGTATTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAGAGTTGGTAGCTCTTGATCCG  
GCAACAAACCAACCGCTGGTAGCGGTGTTTGTGTTGCAAGCAGCAGATTACGCGCAGAAAGGATCTC  
AAGAAGATCCTTTGATCTTTCTACGGGGTCTGACGCTCAGTGGAACGAAACTCACGTTAAGGGATTTTGGTCA  
TGAGATTATCAAAAAGGATCTTCAACCTAGATCCTTTTAAATTAATAATGAAGTTTAAATCAATCTAAAGTATAT  
ATGAGTAAACTTTGGTCTGCACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGGATCTGTCTATTTTCGTT  
CATCCATAGTTGCCCTGACTCCCGTCCGTGCTAGATACTACGATACGGGAGGCTTACCATCTGGCCCCAGTGTG  
CAATGATACCGGAGACCCACGCTCACCGGCTCCAGATTATCAGCAATAAACAGCCAGCCGGAAGGCCGAGC  
GCAGAAAGTGCTCCTGCAACTTTATCCGCCCTCCATCCAGTCTATTAAATTGTTGCCGGAGCTAGAGTAAGTAGTT  
CGCCAGTTAAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGCAGCTCGTCTGTTGGTATGG  
CTTCATTCAAGTCCGGTTCCCAACGATCAAGCGAGTTACATGATCCCCCATGTTGTGCAAAAAGCGGTTAGCT  
CCTTCGGTCCCTCCGATCCGTTGTCAGAAAGTAAGTTGGCCGCGAGTGTATCACTCATGGTTATGGCAGCACTGCATA  
ATTCTCTTACTGTCAATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCAATCTGAGAAT  
AGTGATGCGGACCGAGTTGCTCTTGCCCGCGCTCAATAACGGGATAATACCGGCCACATAGCAGAACTTTAA  
AAGTGCTCATCATGGAAAACGTTCTTCGGGGCGAAAACCTCAAGGATCTTACCGCTGTGAGATCCAGTTCCGA  
TGTAACCCACTCGTGCAACCAACTGATCTTCAGCATCTTTTACTTTTACCAGCGTTTCTGGGTGAGCAAAAACAG  
GAAGGCAAAAATGCCGCAAAAAGGGAAATAAGGGCGACACGGAAATGTTGAATACTCATCTCTTCTTTTCAAT  
ATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAA  
TAGGGGTTCCGCGCACATTTCCCCGAAAAGTGC

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## FIG. 6a

```

1  CTGTGGAATG TGTGTAGTT AGGGTGTGGA AAGTCCCAG GCTCCCAGC AGGCAGAAGT
61  ATGCAAAGCA TGCATCTCAA TTAGTCAGCA ACCAGTGTG GAAAGTCCC AGGCTCCCCA
121 GCAGGCAGAA GTATGCAAAG CATGCATCTC AATTAGTCAG CAACCATAGT CCCGCCCTA
181 ACTCCGCCCA TCCCGCCCCT AACTCCGCC AGTTCGCCC ATTCTCCGC CCATGGCTGA
241 CTAATTTTTT TTATTTATGC AGAGCCGAG GCCGCTCGG CCTCTGAGT ATTCCAGAAG
301 TAGTGAGGAG GCTTTTGTGG AGGCTAGGC TTTTGCAAAA AGCTTGCAATG CCTGCAGGTC
361 GGCCGCCACG ACCGGTGCCG CCACCATCC CTGACCCACG CCCCTGACCC CTCACAAGGA
421 GACGACCTTC CATGACCGAG TACAAGCCA CGGTGCGCCT CGCCACCCG GACGACGTCC
481 CCCGGGCCGT ACGACCTC CCGCCACATC GAGCGGTCA CCGAGCTGCA AGAACTCTTC CTCACGCGCG
541 TCGACCCGGA CCGGCTCGA CATGGCAAG GTGTGGTGC CGGACGACGG CGCCGCGGTG GCGGTCTGGA
601 CCACGCCGGA GAGGTCGAA TTCCGGCTG GCCGCGAGC AACAGATGGA AGGCTCTCTG GCGCCGCACC
661 AGTTGAGCGG TTCCGGCTG GCCGCGAGC TTCTGGCCA CCGTCGGCGT CTCGCCCGAC CACCAGGGA
721 GGCCCAAGGA GCGGCTGGG CAGCGCCGTC GGAGACCTCC GCGCCCGCA ACCTCCCCTT CTACGAGCGG GCGGGGTGC
781 AGGTGAGCGG TTCCGGCTG GCCGCGAGC TTCTGGCCA CCGTCGGCGT CTCGCCCGAC CACCAGGGA
841 AGGTGAGCGG TTCCGGCTG GCCGCGAGC TTCTGGCCA CCGTCGGCGT CTCGCCCGAC CACCAGGGA
901 AGGTGAGCGG TTCCGGCTG GCCGCGAGC TTCTGGCCA CCGTCGGCGT CTCGCCCGAC CACCAGGGA
961 AGGTGAGCGG TTCCGGCTG GCCGCGAGC TTCTGGCCA CCGTCGGCGT CTCGCCCGAC CACCAGGGA
1021 AGGTGAGCGG TTCCGGCTG GCCGCGAGC TTCTGGCCA CCGTCGGCGT CTCGCCCGAC CACCAGGGA
1081 AGGTGAGCGG TTCCGGCTG GCCGCGAGC TTCTGGCCA CCGTCGGCGT CTCGCCCGAC CACCAGGGA
1141 AGGTGAGCGG TTCCGGCTG GCCGCGAGC TTCTGGCCA CCGTCGGCGT CTCGCCCGAC CACCAGGGA
1201 AGGTGAGCGG TTCCGGCTG GCCGCGAGC TTCTGGCCA CCGTCGGCGT CTCGCCCGAC CACCAGGGA

```

A

A

## FIG. 6b

A	A
1261	AACTTGTTA TTGCAGCTTA TAATGGTTAC AAATAAAGCA ATAGCATCAC AAATTCACA
1321	AAATAAGCAT TTTTTCACAT TTTTTCACAT GCATTCCTAGT TGTGGTTTGT CCAAACTCAT CAATGTATCT
1381	TATCATGTCT GGATCCCCAG GAAGTCCTC TGTGTCTCA TAAACCCCTAA CCTCCTCTAC
1441	TTGAGAGGAC ATTCCAATCA TAGGCTGCCC ATCCACCCCTC TGTGTCTCTC TGTAAATTAG
1501	GTCACCTTAAC AAAAAGGAAA TTGGGTAGGG GTTTTTCACA GACCGCTTTC TAAGGGTAAT
1561	TTTAAATAT CTGGGAAGTC CCTTCCACTG CTGTGTCCA GAAGTGTGG TAAACAGCCC
1621	ACAAATGTCA ACAGCAGAAA CATACAAGCT GTCAGCTTTG CACAAGGCC CAACACCCCTG
1681	CTCATCAAGA AGCACTGTGG TTGCTGTGTT AGTAATGTGC AAAACAGGAG GCACATTTTC
1741	CCCACCTGTG TAGGTTCCAA AATATCTAGT GTTTTTCATT TTAAGTTGGAT CAGGAACCCA
1801	GCACTCCACT GGATAAGCAT TATCCTTATC CAAACAGCC TTGTGGTCAG TGTTTCATCTG
1861	CTGACTGTCA ACTGTAGCAT TTTTGGGGT TACAGTTGA GCAGGATATT TGGTCTCTGA
1921	GTTTGCTAAC ACACCCCTGCA GCTCCAAAGG TTCCCCACCA ACAGCAAAA AATGAAAATT
1981	TGACCCCTGA ATGGGTTTTC CAGCACCAT TTTTCATGAGTT TTTTGTGTCC CTGAATGCAA
2041	GTTTAAACATA GCAGTTACCC CAATAACCTC AGTTTAAACA GTAAACAGCTT CCCACATCAA
2101	AATATTTCCA CAGGTTAAGT CCTCATTTAA ATTAGGCAA GGAATCTTG AAGACGAAAG
2161	GGCCTCGTGA TACGCCTATT TTTATAGGTT AATGTCATGA TAATAATGGT TTCTTAGACG
2221	TCAGGTGGCA CTTTTCGGG AAATGTGCGC GGAACCCCTA TTTGTTTATT TTTCTAAATA
2281	CATTCAAATA TGTATCCGCT CATGAGACAA TAACCCCTGAT AAATGCTTCA ATAATATGA
2341	AAAAGGAAGA GTATGAGTAT TCAACATTTC CGTGTGCGCC TTATTCCTT TTTTGGCGCA
2401	TTTTGCTTC CTGTTTTTGC TCACCCAGAA ACGTGGTGA AAGTAAAGA TGCTGAAGA
2461	CAGTTGGGTG CACGAGTGG TTACATCGAA CTGGATCTCA ACAGCGGTAA GATCCTTGAG
2521	AGTTTTCGCC CCGAAGAACG TTTTCCAATG ATGAGCACTT TTAAAGTTCT GCTATGTGGC
B	B

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## FIG. 6c

B  
 2581 GCGGTATTAT CCCGTGTTGA CGCCGGGCAA GAGCAACTCG GTCGCCGCAT ACACTATTCT  
 2641 CAGAAATGACT TGGTTGAGTA CTCACCAGTC ACAGAAAAGC ATCTTACGGA TGGCATGACA  
 2701 GTAAGAGAAT TATGCAGTGC TATGCAGTGC GAAGGAGCTA ACCGCTTTT TGCACAACAT GGGGATCAT  
 2761 CTGACAACGA TCGGAGGACC TTGATCGTTG GGAACCGGAG CTGAATGAAG CCATACCAAA CGACGAGCGT  
 2821 GTAACTCGCC TGCCTGCAGC AATGGCAACA ACGTTGCCGA AACTATTAACT TGGCGAACTA  
 2881 GACACCAACGA CTTCCCGGCA ACAATTAAATA GACTGGATGG AGCGGATAA AGTGCAGGA  
 2941 CTTACTCTAG GCTCGGCCCT TCCGGCTGGC CATGCGAGCA CTGGGGCCAG ATGGTAAGCC CTCCCCTATC  
 3001 CCACTTCTGC GTCGCGGTAT ACACGACGGG GAGTCAGGCA ACTATGGATG AACGAAATAG ACAGATCGCT  
 3061 GAGCGTGGGT CTGAGTTATCT GAGATAGGTG CCTCACTGAT TAAGCATTTG TCATTTTAA TTTAAAGGA TCTAGGTGAA GATCCTTTT  
 3121 GTAGTTATCT ACACGACGGG CCTCACTGAT ATTTAAAACT TGACCAAAAT CCCTTAACGT GAGTTTTCGT TCCACTGAGC GTCAGACCCC  
 3181 GAGATAGGTG CCTCACTGAT ATTTAAAACT TGACCAAAAT CCCTTAACGT GAGTTTTCGT TCCACTGAGC GTCAGACCCC  
 3241 CTTAGATTG ATTTAAAACT TGACCAAAAT CCCTTAACGT GAGTTTTCGT TCCACTGAGC GTCAGACCCC  
 3301 GATAATCTCA TGACCAAAAT CCCTTAACGT GAGTTTTCGT TCCACTGAGC GTCAGACCCC  
 3361 GTAGAAAAGA TCAAGGATC TTCTTGAGAT CCTTTTTCCT TCGCGGTAAT CTGCTGCTTG  
 C

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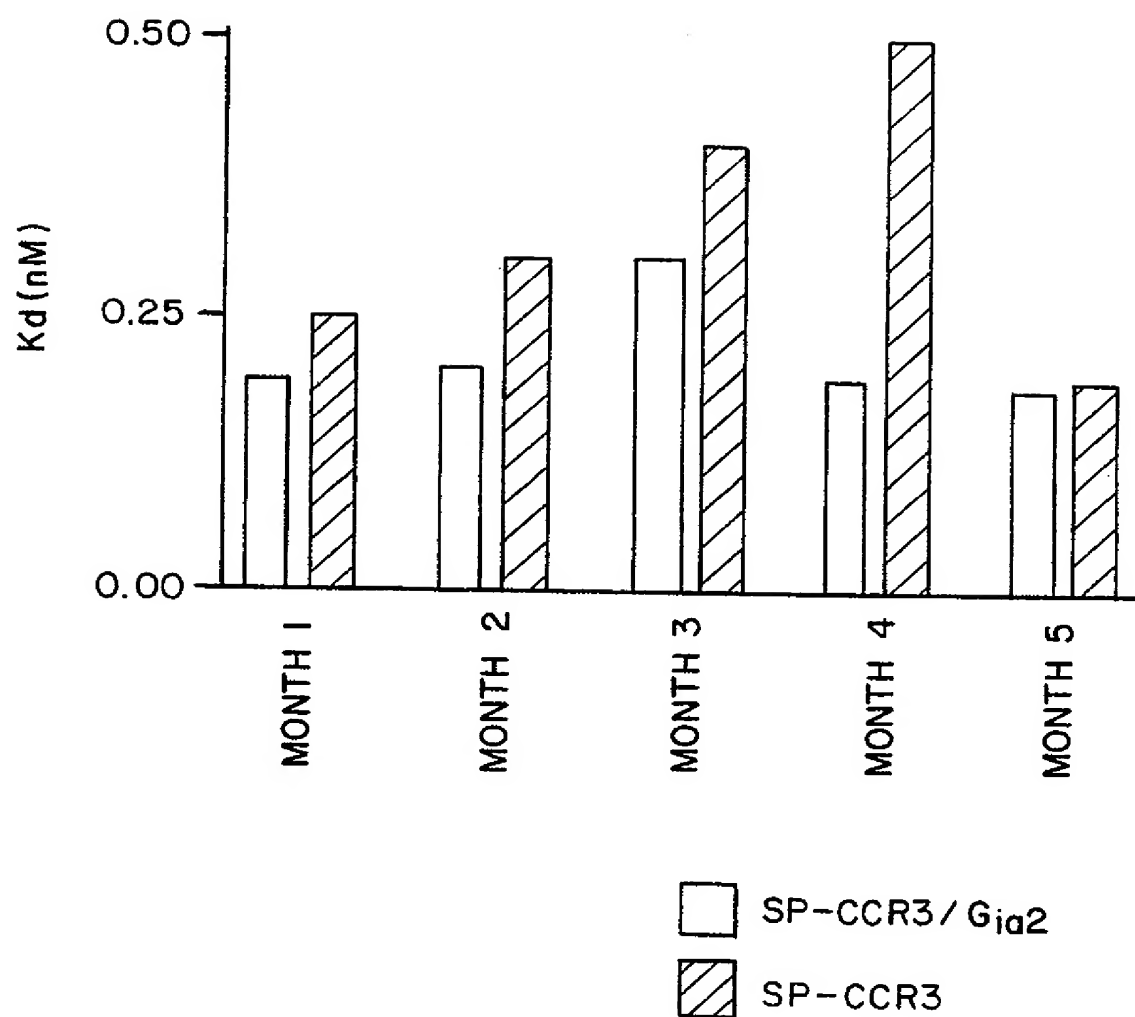
## FIG. 6d

C \_\_\_\_\_ C

3421	CAAAACAAA	AACCACCGCT	ACCAGCGGTG	GTTTGTTTGC	CGGATCAAGA	GCTACCAACT
3481	CTTTTCCGA	AGGTAAGTGG	CTTCAGCAGA	GCGCAGATAC	CAAATACTGT	CCTTCTAGTG
3541	TAGCCGTAGT	TAGGCCACCA	CTTCAAGAAC	TCTGTAGCAC	CGCCTACATA	CCTCGCTCTG
3601	CTAATCCTGT	TACCAGTGGC	TGCTGCCAGT	GGCGATAAGT	CGTGTCTTAC	CGGGTTGGAC
3661	TCAAGACGAT	AGTTACCGGA	TAAGGCGCAG	CGGTCGGGCT	GAACGGGGGG	TTCGTGCACA
3721	CAGCCAGCT	TGGAGCGAAC	GACCTACACC	GAACTGAGAT	ACCTACAGCG	TGAGCTATGA
3781	GAAAGCGCCA	CGCTTCCCGA	AGGAGAGAAAG	GCGGACAGGT	ATCCGGTAAG	CGGCAGGGTC
3841	GGAACAGGAG	AGCGCACGAG	GGAGCTTCCA	GGGGAAACG	CCTGGTATCT	TTATAGTCCT
3901	GTCGGGTTTC	GCCACCTCTG	ACTTGAGCGT	CGATTTTGT	GATGCTCGTC	AGGGGGGGCG
3961	AGCCTATGGA	AAAACGCCAG	CAACGCGGCC	TTTTTACGGT	TCCTGGCCCTT	TTGCTGGCCT
4021	TTTGCTCACA	TGTTCTTTCC	TGCGTTATCC	CCTGATTCTG	TGGATAACCG	TATTACCGCC
4081	TTTGAGTGAG	CTGATACCGC	TCGCCGCAGC	CGAACGACCG	AGCGCAGCGA	GTCAGTGAGC
4141	GAGGAAGCGG	AAGAGCGCCT	GATGCGGTAT	TTTCTCCTTA	CGCATCTGTG	CGGTATTTC
4201	CACCGCATAT	GGTGCACTCT	CAGTACAATC	TGCTCTGATG	CCGCATAGTT	AAGCCAG

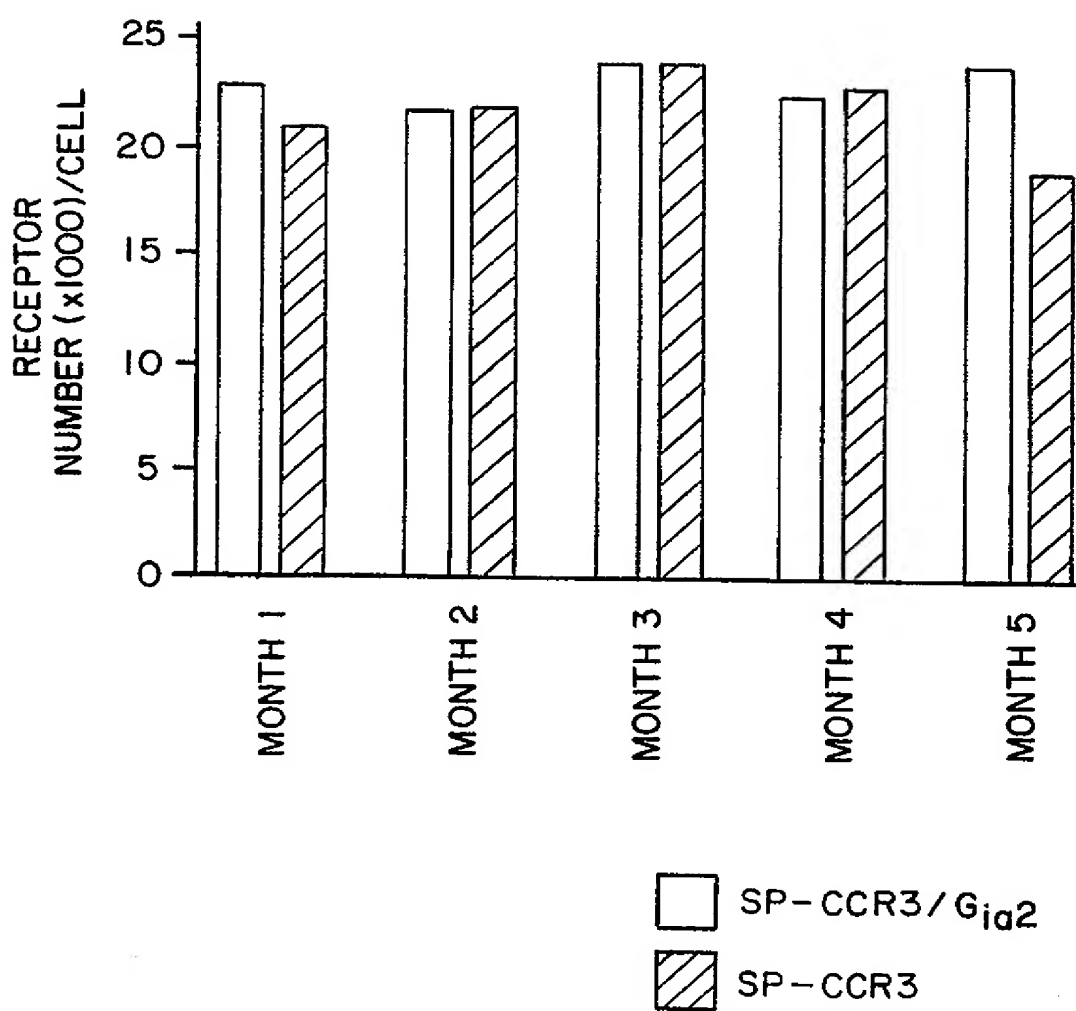
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FIG. 7



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FIG. 8





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FIG. 9

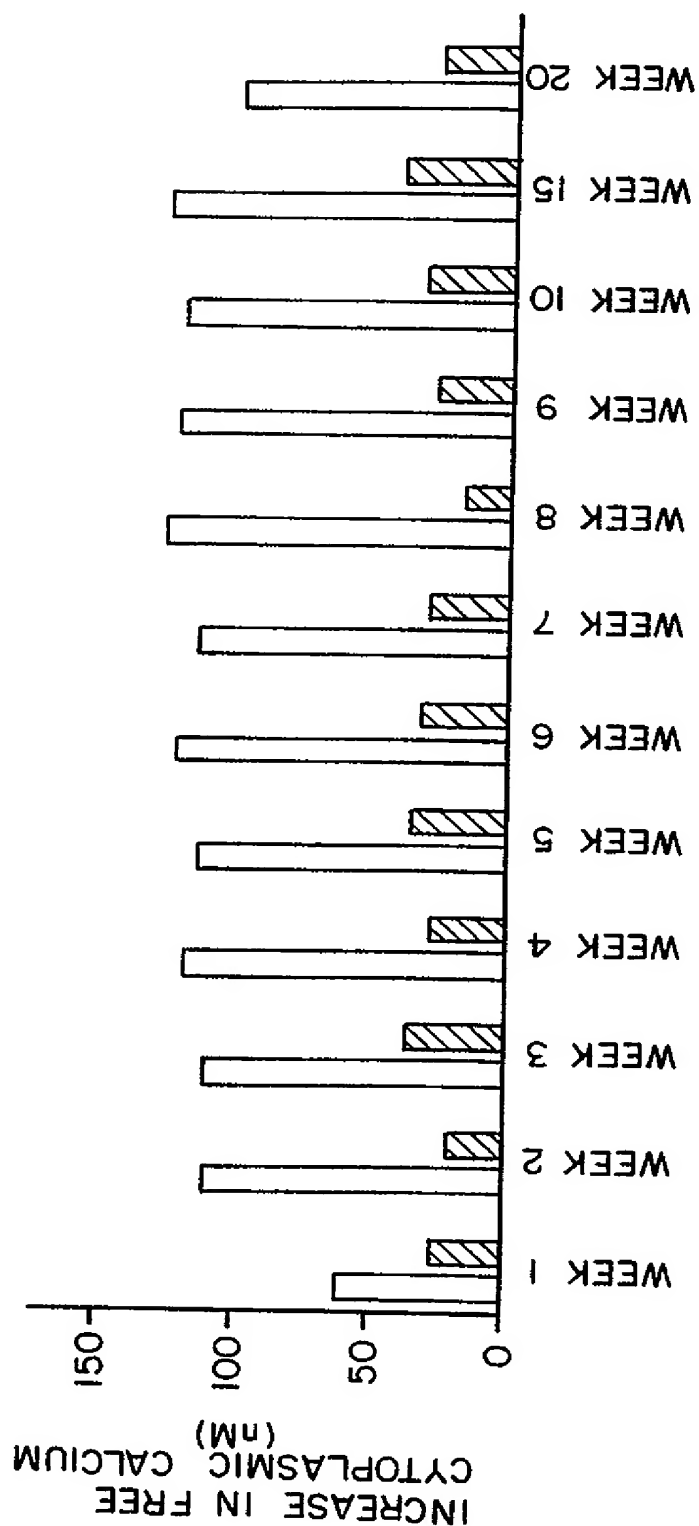
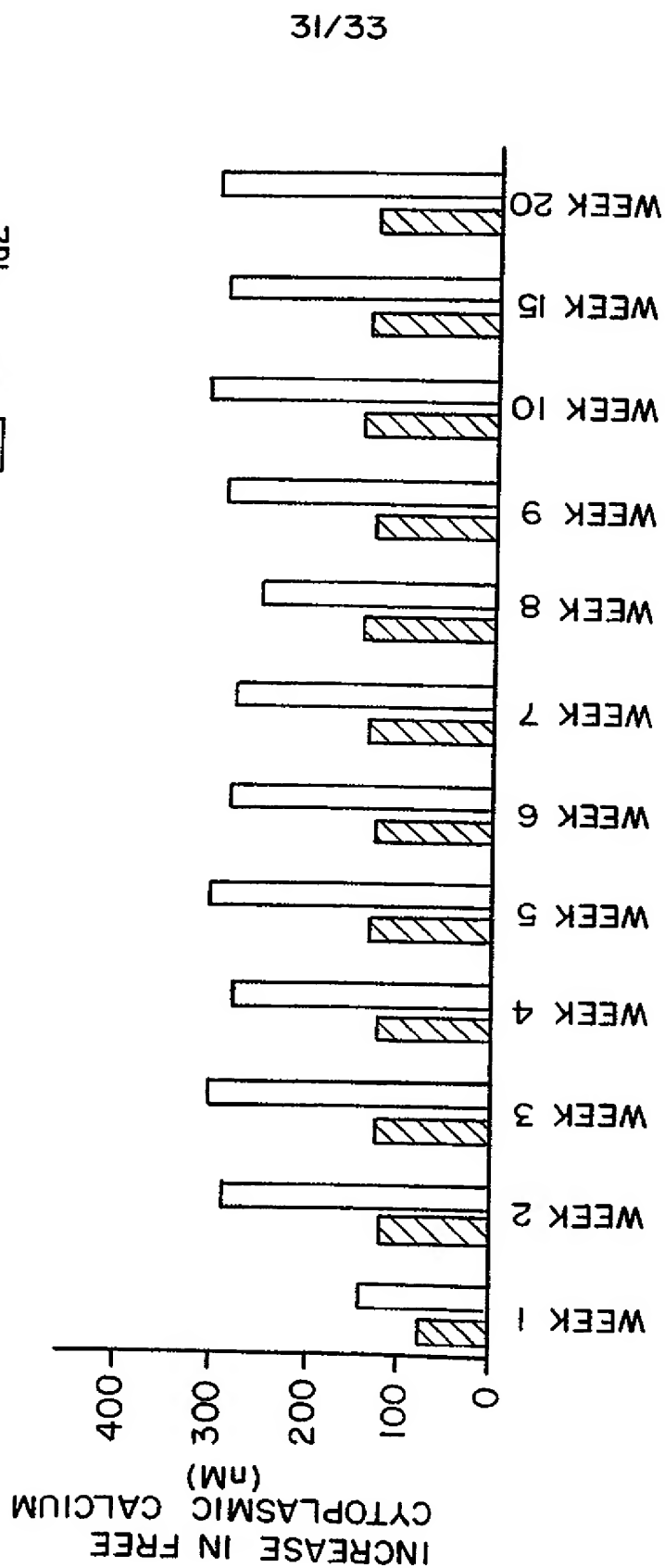
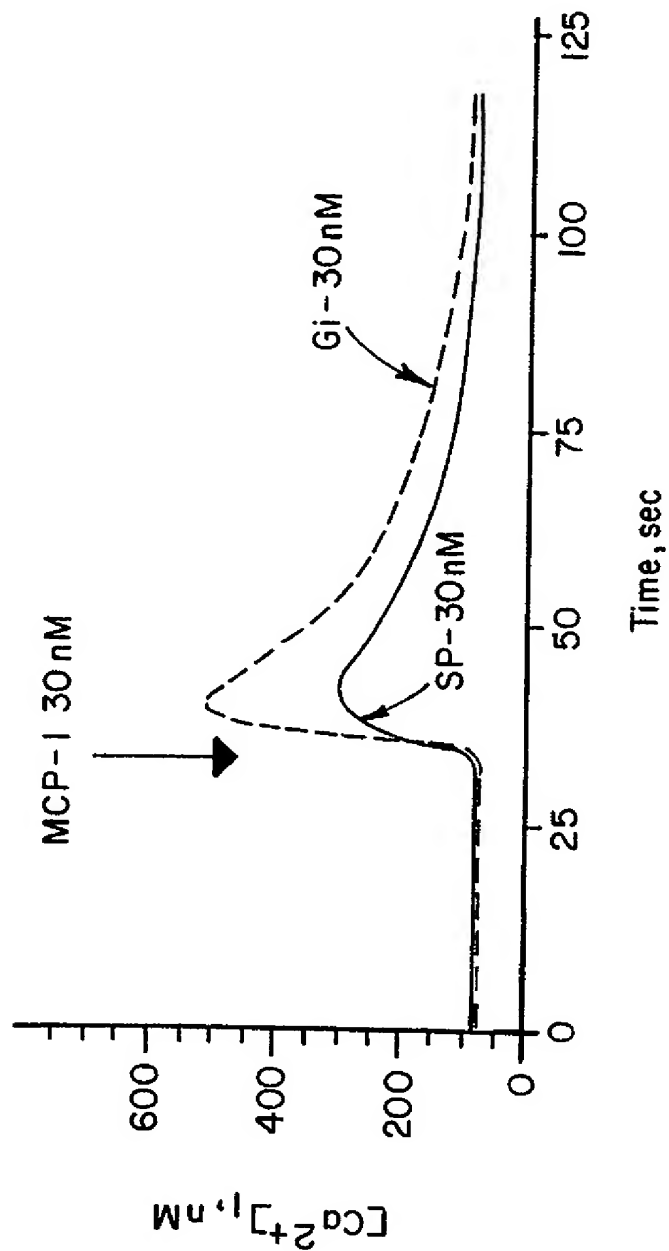


FIG. 10



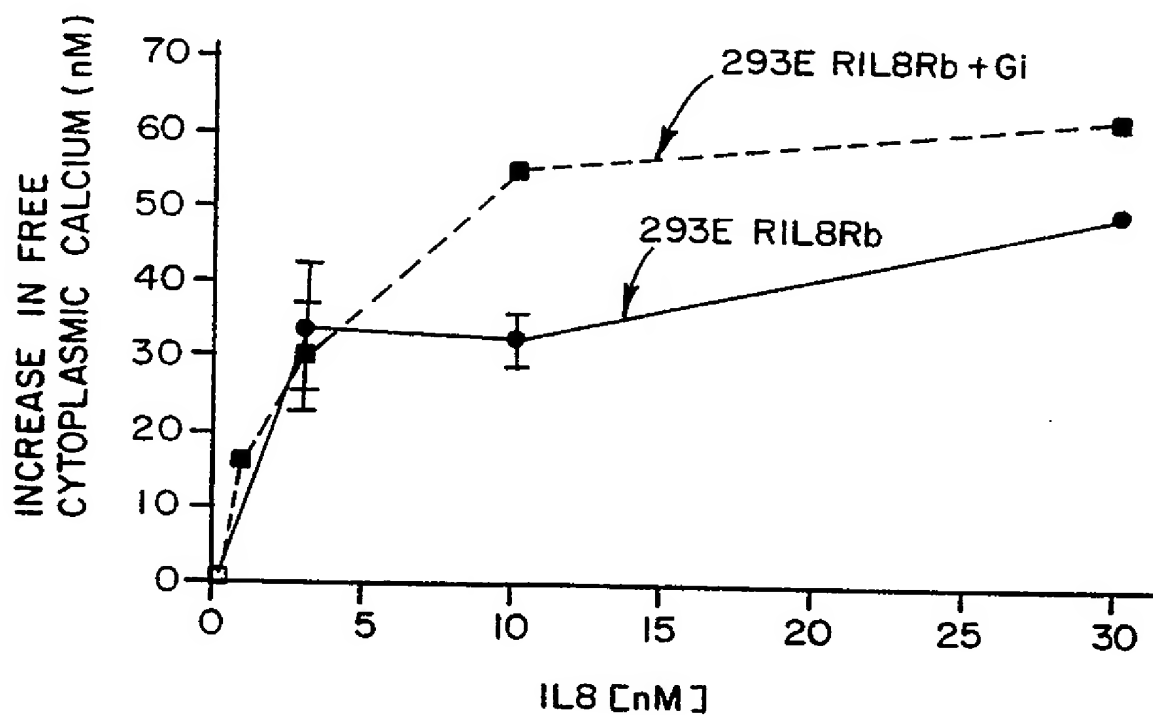
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FIG. 11



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FIG. 12



## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/02852

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : G01N 33/50, 33/52; C07K 14/705; C12N 15/00, 15/12

US CL : 435/7.2, 69.1, 320.1; 536/23.1; 530/402

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.2, 69.1, 320.1; 536/23.1; 530/402

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KUANG, Y. et al. Selective G Protein Coupling by C-C Chemokine Receptors. J. Biol. Chem. 23 February 1996, Vol. 271, No. 8, pages 3975-3978, see entire document.	1-35
Y	HERRLICH, A et al. Involvement of Gs and Gi Proteins in Dual Coupling of the Lutening Hormone Receptor to Adenylyl Cyclase and Phospholipase C. J. Biol. Chem. 12 July 1996, Vol. 271, No. 28, pages 16764-16772, see entire document.	1-35
Y	DAMAJ, B. B. et al. Physical Association of Gi2 Alpha with Interleukin-8 Receptors, J. Biol. Chem. 31 May 1996, Vol. 271, No. 22, pages 12783-12789, see entire document.	1-35

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

28 MAY 1999

Date of mailing of the international search report

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International application No.  
PCT/US99/02852

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	DICKENSON, J. M. et al. Activation of Phospholipase C by G-protein Beta-gamma-Subunits in DDT1MF-2 Cells. E. J. Pharmacol. 1995, Vol. 288, No. 3, pages 393-398, see entire document.	1-35
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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/02852

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BOYER, J. L. et al. Selective Activation of Phospholipase C by Recombinant G-Protein Alpha- and Beta-gamma-Subunits. J. Biol. Chem. 28 January 1994, Vol 269, No. 4, pages 2814-2819, see entire document.	1-35
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Y	DUZIC, E. et al. Factors Determining the Specificity of Signal Transduction by Guanine Nucleotide-binding Protein-Coupled Receptors. J. Biol. Chem. 15 May 1992, Vol 267, No. 14, pages 9844-9851, see entire document.	1-35
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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/02852

### B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, MEDLINE, JAPIO, BIOSIS, SCISEARCH, WPIDS

Search terms: g protein, calcium, gi, gia, gia2, gia3, gi1, gi2 gi3, chemokine, interleukin, fluorescence, fura, indo, fluo, phosphorylase C, episomal